Bone Marrow-Specific *Sirt6* Deficiency Enhances Atherosclerosis by Increasing Macrophage Scavenger Receptor 1 Expression

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1. SUMMARY

1.1 Summary In German

Hintergrund: Der auf Makrophagen exprimierte "Scavenger" Rezeptor 1 (Msr1) vermittelt die Aufnahme modifizierter low-density Lipoproteine (mLDL) durch Makrophagen, ein entscheidender Schritt in der Entstehung von Atherosklerose. Sirt6, eine nukleäre NAD^+-abhängige Deacetylase, aktiviert durch kalorische Restriktion, hat eine protektive Funktion gegen alters-assoziierte Erkrankungen.

Methoden und Resultate: Heterozygote Sirt6 Deletion hatte keinen Einfluss auf Atherosklerose in ApoE-/- Mäusen unter cholesterin-reicher Diät (high cholesterol diet, HCD) während 12 Wochen. Knochenmarks-spezifische Sirt6 Deletion andererseits erhöhte die Ausbildung aortaler, atherosklerotischer Plaques in ApoE-/- Mäusen unter HCD. Aortenwurzel-Schnitte von Sirt6-defizienten, Knochenmarks-transplantierten Mäusen wiesen erhöhte Lipid-Konzentrationen, Makrophagen-Zahlen, VCAM-1 Expression und Msr1 Levels auf Makrophagen auf. Plasma Lipide derweil waren vergleichbar zwischen den Gruppen. In RAW 264.7 Makrophagen, erhöhte Sirt6 Knockdown die Aufnahme Dil-markierten oxLDL's , während Sirt6 Überexpression diese reduzierte; Silencing von Sirt6 erhöhte die Expression von Msr1 auf mRNA- und Protein-Ebene, während die Expression anderer Lipoprotein Aufnahme- und Efflux-Rezeptoren auf Makrophagen unverändert blieb. Ein kombinierter Knockdown von Sirt6 und Msr1 in RAW 264.7 Zellen verhinderte eine gesteigerte oxLDL Aufnahme. Durchfluss-zytometrische Analyse von Makrophagen aus aortalen Plaques Sirt6-defizienter Knochenmarks-transplantierter Mäuse zeigte eine erhöhte Msr1 Expression. Vergleichbare Resultate fanden sich bei

Knochenmarks-Zellen, während die Untersuchung peripherer Monozyten keine Unterschiede in der Msr1-Expression ergab.

Schlussfolgerungen: Der Verlust von Sirt6 in Knochenmarks-derivierten Makrophagen begünstigt Atherosklerose durch eine Erhöhung der Msr1 Expression und erhöhte oxLDL Aufnahme durch Makrophagen. Diese Ergebnisse weisen auf eine Makrophagen-spezifische, protektive Rolle von Sirt6 hin.

1.2 Summary In English

Background: Macrophage Scavenger Receptor 1 (Msr1) mediates the uptake of modified low-density lipoproteins (mLDL) by macrophages, a critical step in atherogenesis. Sirt6 is a nuclear NAD^+-dependent deacetylase that is activated upon caloric restriction and provides protection in age-related diseases.

Methods and Results: Heterozygous Sirt6 deletion did not affect atherosclerosis in *Apoe*$^{-/-}$ mice fed a high-cholesterol diet (HCD) for 12 weeks. However, bone marrow (BM)-specific Sirt6 deletion increased aortic plaque formation in *Apoe*$^{-/-}$ mice on a HCD. Aortic root sections from Sirt6-deficient BM-transplanted mice revealed an increase in lipid content, macrophage numbers, Vcam1 expression and macrophage Msr1 levels. Plasma lipids were similar between the groups. In RAW 264.7 macrophages, Sirt6 knockdown enhanced Dil-labelled oxLDL uptake, whereas Sirt6 overexpression reduced it; silencing of Sirt6 increased mRNA and protein expression of Msr1, whereas expression of other macrophage lipoprotein uptake receptors and cholesterol efflux transporters remained unchanged. Double knockdown of Sirt6 and Msr1 in RAW 264.7 cells abolished the increase in oxLDL uptake. Flow cytometric analysis of macrophages from aortic plaques of Sirt6-deficient BM-transplanted mice showed increased Msr1 expression; similar results were found in BM cells; however, analyses in blood monocytes showed no difference in Msr1 expression.

Conclusions: Loss of *Sirt6* in BM-derived macrophages is proatherogenic through an increase in Msr1 expression and enhanced uptake of oxLDL by macrophages. These findings suggest a macrophage-specific atheroprotective role of Sirt6.

2. ABBREVIATIONS

acLDL: Acetylated LDL

ACS: Acute coronary syndromes

BAT: Brown adipose tissue

BER: Base excision repair

BM: Bone marrow

CCL5: CC-chemokine ligand 5

CNS: Central nervous system

CXCL1: CXC-chemokine ligand 1

DSB: Double-strand break

ER: Endoplasmic reticulum

Fat: Fatty acid translocase

HCD: High cholesterol diet

HDL-c: High-density lipoprotein cholesterol

HFD: High-fat diet

HIF1α : Hypoxia inducible factor 1α

H_2O_2: Hydrogen peroxide

ICAM1: Intercellular adhesion molecule 1

IR: Ionizing radiation

KO: Knockout

LDL-c: Low-density lipoprotein cholesterol

LDLR: Low density lipoprotein receptor

LFA1: lymphocyte function-associated antigen 1

LOX1: Lectin-like oxidized LDL receptor 1

LRP-1: Low-density lipoprotein receptor-related protein 1

MARCO: Macrophage receptor with collagenous structure

MEF: Mouse embryonic fibroblasts

MerTK: Receptor tyrosine kinase

mLDL: Modified low-density lipoproteins

MMS: Methylmethanesulfonate

Msr1: Macrophage scavenger receptor 1

NA: Nicotinic acid

NAM: Nicotinamide

NMN: Nicotinamide mononucleotide

NMNAT: Nicotinamide mononucleotide adenylyltransferase

NAMPT: Nicotinamide phosphoribosyltransferase

NR: Nicotinamide riboside

oxLDL: Oxidized LDL

Pcsk9: Proprotein convertase subtilisin/kexin type 9

PRR: Pattern recognition receptors

Pfk-1: Phosphofructokinase-1

ROS: Reactive oxygen species

SMC: Smooth muscle cells

SR: Scavenger receptors

SR-B1: Scavenger receptor B1

Srebp: Sterol-regulatory element binding protein

SREC1: Scavenger receptor expressed by endothelial cells 1

STAC: Sirtuin activating compounds

TCA: Tricarboxylic acid

Tpi: Triose phosphate isomerase

TNF-α: Tumor necrosis factor α

VCAM1: Vascular cell adhesion molecule 1

VLA4: Very late antigen 4

VSMC: Vascular smooth muscle cells

3. INTRODUCTION

3.1 Atherosclerosis and foam cell formation

Atherosclerosis is the leading cause of cardiovascular disease and mortality in Western countries [1]. The initiation of atherosclerosis involves the accumulation of oxidized lipids within the endothelium of the arterial walls, which progresses to form atherosclerotic lesions or plaques – the hallmark of the disease. Atherosclerotic plaques consist of asymmetrical plaque thickenings within the innermost sub-endothelial layer of the artery, the intima [2]. In the presence of excess circulating LDL-cholesterol (LDL-c), increased LDL-c is taken up within the endothelial cells where it undergoes modifications like oxidation by free radicals from reactive oxygen species, thereby being transformed into pro-inflammatory particles namely oxidized LDL (oxLDL) [3]. These protein modifications enhance the binding of LDL to extracellular matrix components and result in the increased sub-endothelial retention of oxLDL. oxLDL induces expression of adhesion molecules in endothelial cells and stimulates vascular smooth muscle cells (VSMCs) to release chemokines and chemo-attractants, leading to the recruitment of monocytes and T cells (as shown in Figure 1). Upon tissue infiltration, monocytes become activated and differentiate to macrophages. These monocyte-derived macrophages are recruited by endothelial chemokines and adhesion molecules expression, and they phagocytose the native and modified LDL (mLDL) through Fc- and scavenger receptors to become foam cells [4].

These atherosclerotic lesions expand over time and result in the narrowing of the arterial lumen. If these events occur in coronary arteries of patients, relevant

narrowing may lead to angina pectoris, but this is rarely fatal in the absence of scarring of the myocardium [5]. The susceptibility of a coronary plaque to rupture and form a blood clot (thrombus) with total artery occlusion is the most frequent cause of acute coronary syndromes (ACS) [6]. Histological analyses of atherosclerotic thrombi have shown that more than 40% of the volume of most disrupted plaques contain a necrotic lipid core, consisting of lipids, cholesterol crystals and necrotic debris surrounded by a thin fibrous cap [7].

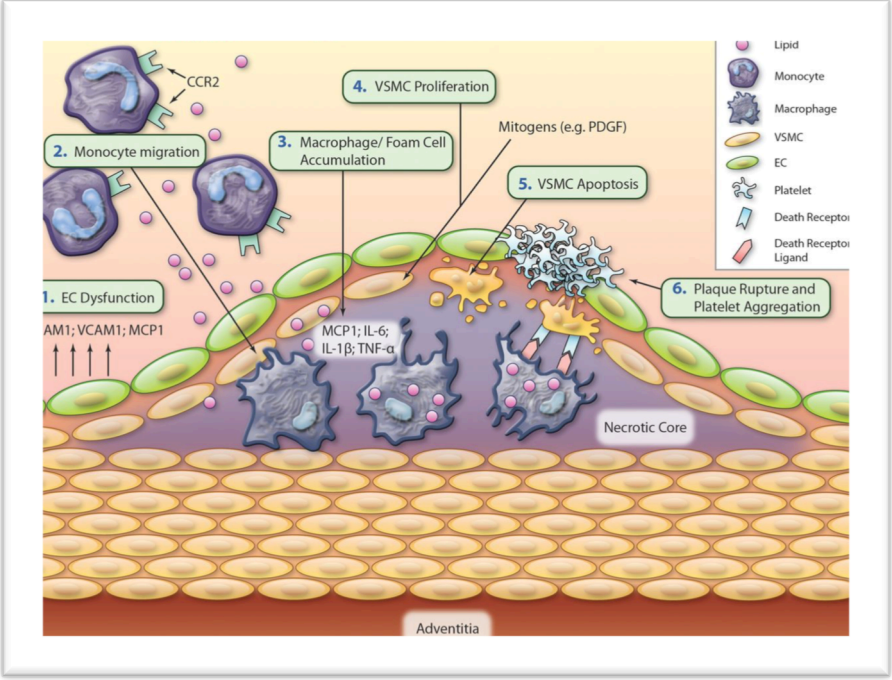

Figure 1: Step-wise scheme of atherogenesis and plaque rupture: The first event in the plaque formation is endothelial dysfunction characterised by activation of adhesion molecules like ICAM-1 and VCAM-1 (Step 1). This facilitates the attachment and rolling of circulating monocytes and their entry into the subendothelial space (Step 2). The monocytes differentiate to form macrophages, which engulf and accumulate the modified lipids to form foam cells (Step 3). The vascular smooth muscle cells (VSMC) proliferate and synthesize collagen that forms the fibrous cap enclosing the lipid core (Step 4). Apoptosis of the VSMC and increased matrix metalloproteinase results in fibrous cap thinning (Step 5). This is followed by plaque rupture, with subsequent platelet attachment and thrombosis (Step 6). (Adapted from Wang JC, Bennett M, Circulation Research, 2012) [8].

3.2 Role of macrophages in the pathogenesis of atherosclerosis

At the onset, the uptake of lipids by macrophages is most likely a normal immune response to lipid accumulation. In this setting most of the excess modified lipids are cleared by means of macrophage phagocytosis. However, in the presence of excess LDL particles the retention of oxLDL particles by macrophages leads to the formation of foam cells. LDL uptake by monocyte-derived macrophages is one of the earliest steps in atherogenesis resulting in foam cells. The damaging modified lipoproteins in the arterial wall are recognized by **pattern recognition receptors** (PRR) on immune cells such as macrophages. PRRs can initiate signaling cascades and innate immune responses. This dysregulation of the local lipid metabolism alters macrophage phenotypes and ensues an inflammatory immune response [9].

Scavenger receptors are one of the main PRRs expressed by macrophages and play an important role in macrophage function and metabolism. They are surface receptors and have a large spectrum of endogenous and exogenous ligands that are endocytosed and undergo lysosomal degradation [10]. They can also recognize unmodified and modified forms of LDL such as oxLDL. Scavenger receptors (SR) include a family of receptors made up of eight classes (A, B, C, D, E, F, G and H) that differ in their ability to target the various modified lipoprotein which act as their ligands (Table 1). Numerous members of the scavenger receptor family — including macrophage scavenger receptor 1 (Msr1; also known as SR-A1*)*, macrophage receptor with collagenous structure (MARCO; also known as SR-A2), CD36 (also known as platelet glycoprotein 4), scavenger receptor B1 (SR-B1), lectin-like oxidized LDL receptor 1 (LOX1), and scavenger receptor expressed by endothelial cells 1

(SREC1) and can take up modified forms of LDL and lead to the formation of foam cells [10].

Table 1. Scavenger Receptor Family, Cell Types, Functions, and Ligands

Class/Name	Cell Type	Function	Major Ligands
Class A			
SRA I/II/III	Macrophages	Uptake of modified LDL, innate immunity	AcLDL, oxLDL, AGE, LPS, LTA
MARCO	Spleen, macrophages	Innate immunity?	AcLDL, bacteria
Class B			
CD36	Platelets, monocytes, macrophages	Fatty acid transporter, uptake of apoptotic cells, Uptake of modified LDL	OxLDL, apoptotic cells
SR-BI (CLA-I)	Adrenals, liver, gonads	Cholesterol transport	HDL, oxLDL
Other			
CD68	Macrophages	Unknown	OxLDL, apoptotic cells
SR-C	Embryonic macrophages	Uptake of apoptotic cells	AcLDL
SREC 1	Endothelial cells	Unknown	AcLDL, oxLDL
LOX-1	Endothelial cells	Unknown	OxLDL

Of all scavenger receptors, the role of Msr1 is the most widely studied in the pathogenesis of atherosclerosis. Msr1 is a trimeric scavenger receptor. In humans and mice Msr1 plays a critical role in oxLDL uptake and foam cell formation. For example, overexpression of human Msr1 gene in mice is associated with increased foam cell formation and cholesterol ester accumulation upon incubation with oxLDL [11]. Msr1 is also highly expressed in atherosclerotic lesions [12]. These early studies have opened several avenues for investigating the role of scavenger receptors and have led to the assignment of Msr1 as an important proatherogenic receptor.

3.3 Class A Macrophage Scavenger receptor 1 (Msr1) in atherogenesis

Since their description in 1979 by Goldstein et al., scavenger receptors have been defined by their ability to 'scavenge' mLDL from their environment for internalization and subsequent degradation [13]. The excess mLDL internalization and retention produces massive oxidation and deposition of intracellular cholesterol. The main ligands of Msr1 are known to be acetyl LDL (acLDL) *in vitro* and oxLDL. Msr1 can bind and uptake both oxLDL and acLDL, leading to an increase in foam cell formation and are thus implicated in the pathological deposition of cholesterol during atherogenesis [14].

The function of Msr1 has been investigated *in vivo* using genetically modified mice. To study the effect of Msr1 deficiency, knockout (KO) mice were generated by the disruption of exon 4 of the Msr1 gene that is critical for the generation of the trimeric functional receptor [14]. Isolation and subsequent oxLDL treatment of resident peritoneal macrophages from Msr1-deficient mice show an 80% reduction in acLDL clearance and a 30% reduction in oxLDL clearance [15]. To study the relevance of Msr1 in atherosclerosis, Msr1 KO mice were crossbred with different mouse strains susceptible to atherosclerosis. On an Apoe-deficient background, Msr1-deficiency induced conflicting results: The first report described a moderate increase in plasma cholesterol levels coinciding with a 40% increase in lesion areas[16]; in contrast, other studies found smaller atherosclerotic lesions [17]. On a LDL receptor (LDLR)-deficient background, the absence of Msr1 results in a 20% lower plasma cholesterol level and only a 20% reduction in atherosclerosis [18]. These findings suggest that the effects of Msr1 on macrophages are highly context-

dependent.

Msr1 is important for the rapid removal of apoptotic cells by macrophages [19]. In addition to having a role in lipid uptake and apoptotic cell clearance, Msr1 also plays a critical role in the process of cell adhesion (Figure 2). In 1993, Fraser et al first elucidated this function of Msr1 by developing a monoclonal antibody (2F8) directed against murine Msr1 receptor and showed that 2F8 inhibited the calcium-independent adhesion of RAW 264 macrophages to tissue culture plastic plates [20]. This was the first evidence that demonstrated that Msr1 could mediate cellular adhesion. Subsequent studies showed that 2F8 could also block the adherence of macrophages in tissues. Peritoneal macrophages from Msr1 KO mice were able to adhere to plastic dishes but the process was slower compared to wild-type macrophages, confirming Msr1 is essential for macrophage adhesion *in vitro* [14]. This effect on adhesion demonstrated a functional relevance of Msr1 in the recruitment and retention of mononuclear phagocytes to tissues, such as endothelial cells and atherosclerotic lesions [17].

Thus, Msr1 acts as a key protagonist of atherogenesis by its involvement in three major steps involved in the initiation and progression of atherosclerosis:

1. Msr1 is a major receptor for the uptake of modified LDL and thus is critical for the formation of macrophage-derived foam cells.
2. Msr1 is involved in removal of apoptotic cells from the atherosclerotic plaque.

3. Msr1 mediates the adherence and interaction of the monocyte-derived macrophages with other cells in the plaque such as endothelial cells, smooth muscle cells, and/or other macrophages.

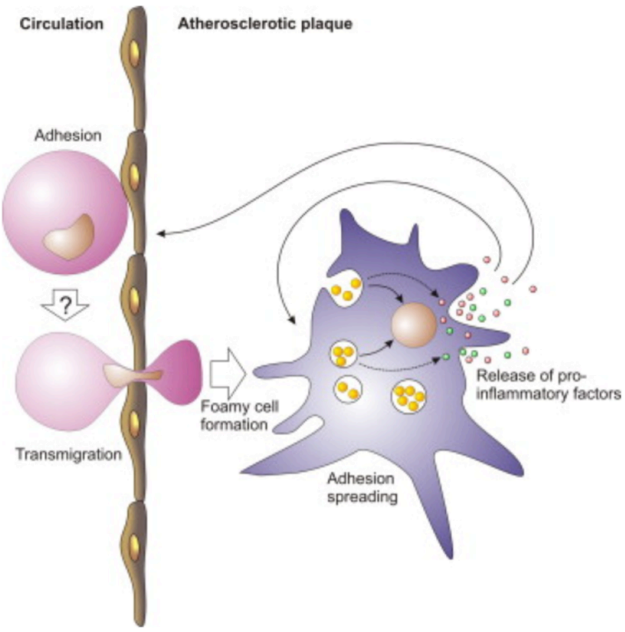

Figure 2. Events regulated by Msr1 during atherogenesis: Msr1 upregulated on circulating monocytes are involved in adhesion of monocytes to activated endothelium and their transmigration into the lesion. Msr1 is important for the release of pro-inflammatory cytokines and mediates the uptake of modified lipoproteins leading to foam cell formation. (From Kzhyshkowska et al., Immunobiology, 2012) [10].

3.4 Role of monocyte subsets in plaque formation

Monocytes originate from bone marrow-derived progenitor cells. Cellular cholesterol content is known to influence early stages of monocyte development and subsequent atherogenesis [21]. The absence of cholesterol efflux transporter-ABCA1 in mouse progenitor cells increases the circulating monocytes and ensuing atherosclerosis [22].

Peripheral blood monocytes are not a homogeneous population (Table 2). Monocyte heterogeneity was first reported with the identification of a minor population of CD16 (FcgRIII)- expressing cells within circulating human monocytes

[23]. Meanwhile three functional subsets of human monocytes have been identified. The new nomenclature that groups human monocytes into three subsets is based on the expression of the surface markers CD14 and CD16 [24]. Based on this nomenclature, the major population of human monocytes (90%) expresses high CD14 but no CD16 expression ($CD14^{++}CD16^{-}$ or $CD14^{+}CD16^{-}$) are termed **classical monocytes**, whereas the minor population of human monocytes (10%) is further sub-divided into the **intermediate monocyte subset**, with low CD16 and high CD14 ($CD14^{++}$ $CD16^{+}$ or $CD14^{+}$ $CD16^{+}$), and the **non-classical monocyte subset**, with high CD16 but relatively lower CD14 expression ($CD14^{+}CD16^{++}$) [24].

The physiological role of the monocyte subsets *in vivo* is not fully elucidated. The different subsets may have different roles during metabolic homeostasis, immune defense/inflammation, and tissue repair, depending on their capacity to become activated and/or secrete inflammatory cytokines in response to different stimuli, antigen processing and presentation. In general terms, both human classical and intermediate monocytes have inflammatory properties (also termed "inflammatory" monocytes) [25], while non-classical monocytes display patrolling properties (also termed "alternative" or "patrolling" monocytes) [25]. Similar to the nomenclature of human monocyte subpopulations, murine monocytes are also sub-divided into $Ly6C^{low}$ and $Ly6C^{hi}$ monocytes, where murine $Ly6C^{hi}$ are the inflammatory subtype and $Ly6C^{low}$ are the alternative/anti-inflammatory monocyte subtype [25].

Recent studies have shown that $Ly6C^{hi}$ monocytes are the precursors of atherosclerotic lesion macrophages [26, 27], while the exact contribution of $Ly6C^{low}$

monocytes remains unclear. Monocytosis in hypercholesterolemic mice is due to an increase in the inflammatory $Ly6C^{hi}$ subset. This subpopulation constitutes the majority of cells recruited to growing atherosclerotic plaques and which is thought to be the source of the M1 macrophages (also known as classically activated macrophages) that are found in the plaques [28].

Table 2. Human and murine monocyte subsets, surface makers and functions

Subset	Markers	Main functions
Human beings		
Classical	$CD14^{++}$ $CD16^{-}$	Phagocytosis, inflammatory effectors
Intermediate	$CD14_{++}$ $CD16^{+}$	Inflammatory effectors
Non-classical	$CD14^{+}$ $CD16^{++}$	Patrolling, antiviral role
Mouse		
$Ly6C^{low}$	$CD11b^{+}$ $CD115^{+}$ $Ly6C^{+}$	Phagocytosis, Inflammatory effectors
$Ly6C^{high}$	$CD11b^{+}$ $CD115^{+}$ $Ly6C^{-}$	Patrolling, tissue repair

Macrophages derived from monocyte precursors undergo specific differentiation depending on the local tissue environment. The activation and differentiation to different macrophage phenotypes depends on the environmental cues within tissues such as damaged cells, cytokines or lymphocytes. The M1 macrophage phenotype is characterized by the production of high levels of pro-inflammatory cytokines in an effort to mediate resistance to pathogens. They have strong microbicidal properties, produce high amounts of reactive nitrogen and oxygen intermediates, and promote Th1 responses. Thus, M1 macrophages are classically activated and implicated in initiating and sustaining inflammation [29]. In contrast, M2 macrophages are characterized by their involvement in parasite control, tissue remodeling, immune regulation, tumor promotion and efficient

phagocytic activity [30]. They decrease inflammation and encourage tissue repair [31].

The polarization states between M1 and M2 are to some extent reversible and dependent on the local milieu in an atherosclerotic vessel. Within the atherosclerotic vessel wall, there is a mixed population of both M1 and M2 macrophages [32]. Macrophages present in the unstable lesion area surrounding the necrotic core consist of mainly M1 macrophages, while macrophages within the fibrous cap surrounding the necrotic core express both M1 and M2 markers [33]. Of note, the M2 macrophages exert beneficial profibrotic and plaque stabilizing effects, which might counteract the potentially deleterious proinflammatory effects of M1 macrophages within the fibrous cap [30, 33].

3.5 Caloric restriction and the *Sir2* connection in worms and yeast

Long term caloric restriction (reduced food consumption without any malnutrition) or intermittent fasting (short-term starvation) has been for a while associated with extended lifespan in yeast, plants, worms, flies and rodents. McCay et al. described for the first time that caloric restriction prolongs mean and maximal lifespan in rats compared with ad libitum feeding [34]. Though this concept was introduced almost 50 years ago, the following impact on research in metabolism, nutrition and aging is compelling and lasting. Caloric restriction has an effect on aging. Its effects are even more important on **healthspan** – i.e. the period of life during which an individual is healthy — by reducing the incidence of metabolic diseases, cancer, atherosclerosis and neurodegeneration [35, 36].

Early studies show that caloric restriction does not alter the metabolic rate in animals [37]. However, later studies demonstrated that if the metabolic rate is normalized to the lean body mass of the animals, there is an increase in caloric restricted animals [38]. This was an important finding because it challenged the idea that the total metabolic activity over the lifetime of the animal was fixed. Caloric restricted animals enjoyed more total metabolic activity adjusted for body weight over their lifetimes than did the ad libitum-fed controls.

Genetic studies in model organisms like *Caenorhabditis elegans*, and *Saccharomyces cerevisiae* suggested that caloric restriction might be a tightly regulated process, with the gene ***Sir2,*** playing an important role. The *Sir2* gene transcript is a NAD^+-dependent histone deacetylase [39] with NAD^+ acting as cofactor for enzyme activity [40, 41] (Figure 3). Several studies confirmed the link between caloric restriction and *Sir2* gene: One study showed caloric restriction does not extend life span of yeast anymore when *Sir2* was deleted [42]. Another study demonstrated increased *Sir2* activity *in vivo* upon caloric restriction [43]. And finally, the *Sir2* enzyme activator, resveratrol, was shown to extend yeast replicative life span [44]. Overall, the activity of *Sir2* suggested that this protein senses the metabolic state of cells and sets the lifespan accordingly [45].

While being a substrate for *Sir2* activity, $NAD^+(H)$ and $NADP^+(H)$ are also essential coenzymes participating in many cellular redox reactions in all living system. Thus, reduced NAD^+ metabolism is linked to many diseases such as cancer, metabolic and neurodegenerative disorders [46]. Although redox reactions do not alter the total cellular NAD^+ content, cellular processes such as *Sir2*-mediated

protein deacetylation consume NAD^+. Hence it is critical to maintain a constant renewal of cellular NAD^+ to maintain cellular metabolism [46]. NAD^+ is synthesized from a number of precursor molecules. The amino acid tryptophan is utilised for the *de novo* biosynthesis of NAD^+. NAD^+ metabolites such as nicotinamide (NAM), nicotinic acid (NA) and nicotinamide riboside (NR) can also be salvaged and re-assimilated into NAD^+ (as shown in figure 3).

NAD^+ levels directly affect Sir2 activity, while on the other hand NADH has been shown to be a competitive inhibitor of Sir2. Lowering NADH levels causes an increase in Sir2 activity [42]. However, NADH is a weak inhibitor of Sir2, and *in vivo* NADH levels are too low to result in the inhibition of Sir2 activity [47]. It is thus likely that the intracellular compartmentalization of NAD^+ and NADH or local protein interactions result in locally high NAD^+/NADH ratios, which activate *Sir2 in vivo*. The dependence of Sir2 activity on the intrinsic cellular levels of NAD^+ and its high K_m values for NAD^+ alludes to the likely link between Sir2 activity and the metabolic homeostasis of a cell [47, 48].

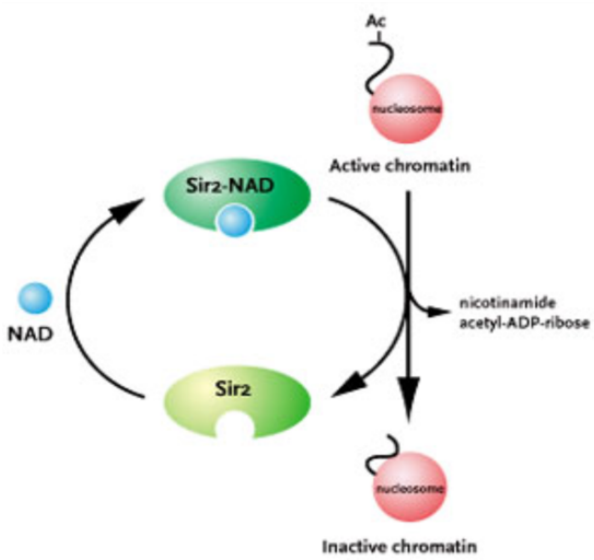

Figure 3: The Sir2 protein requires NAD^+ for its enzymatic activity. It couples NAD^+ breakdown to nicotinamide and ADP-ribose with the removal of acetyl groups from histone and other proteins. The acetyl moiety is transferred to ADP-ribose, creating acetyl-ADP ribose. This Sir2 enzymatic activity links energy metabolism, chromatin silencing and aging.

3.6 Mammalian orthologues of Sir2: Sirtuin family of histone deacetylases

Research that started out in simple laboratory organisms such as *S. Cerevisia and C. Elegans* by the identification of the *Sir2* gene and its contribution to longevity has been extended to mammalian systems in recent years.

The protective effects of *Sir2* on age-related diseases are conserved in mammals. In mammals, the Sir2 orthologues are named **sirtuins.** The sirtuin family comprises of 7 genes, **Sirt1 to Sirt7** which exert different protective effects (as shown in Figure 4). Mammalian sirtuins have varied effects that are regulated by deacetylating numerous substrates and thereby modulating their activity. Sirt1 is the mammalian sirtuin that is most closely related to yeast *Sir2*. It is the most extensively studied sirtuin in mammals and has many beneficial roles in a number of age-related diseases [49],[50].

Sirt1, Sirt6 and Sirt7 are localized in the nucleus of most cell types and have a role in transcriptional regulation through targeting of transcription factors, cofactors or histones. Sirt2 is mostly found in the cytoplasm and is important for cell cycle control. Sirt3, Sirt4 and Sirt5 are primarily mitochondrial proteins and are crucial for oxidative stress pathways and regulating the activities of metabolic enzymes.

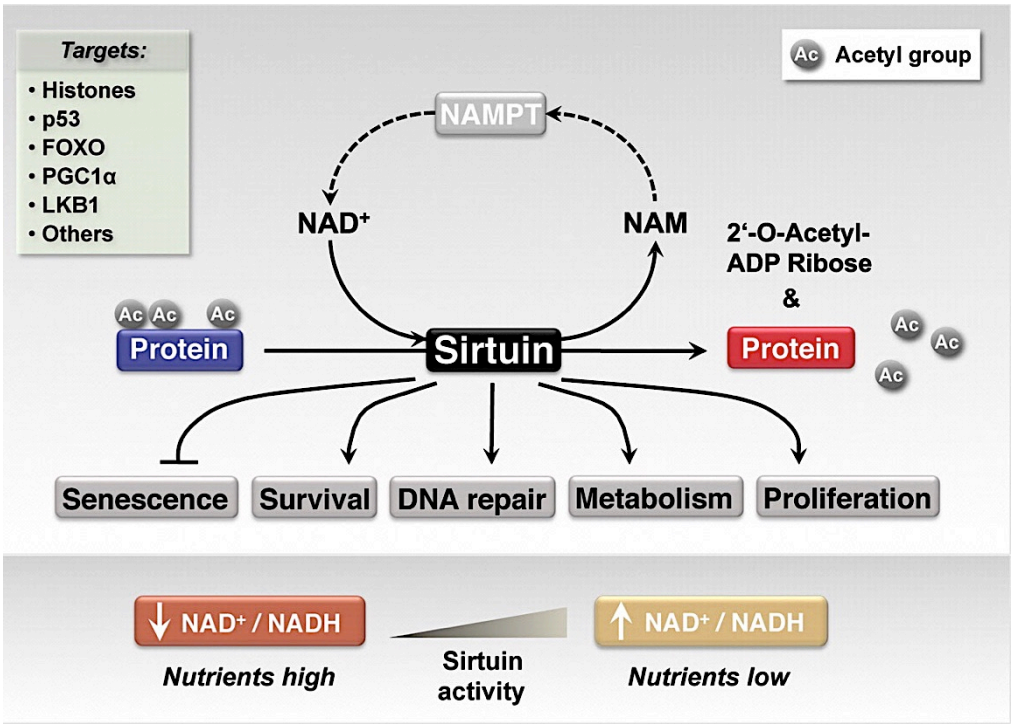

Figure 4: Sirtuins affect multiple diseases. Sirtuins are activated by elevated NAD^+ levels induced during states of increased energy demand such as fasting, calorie restriction and exercise. Sirtuins deacetylate (or ADP-ribosylate) numerous transcription factors, cofactors, histones and enzymes that alter the outputs of metabolic and stress-induced pathways (Adapted from Oellerich and Potente, Circ Res, 2012) [51].

3.7 Role of Sirt6 in aging and life span

Sirt6 is member of the sirtuin family. It is a nuclear protein and functions as a lysine deacetylase, lysine deacylase and an ADP-ribosylase. Deficiency of Sirt6 causes a strong phenotype: Constitutive Sirt6 deletion at the **whole organism level** confers accelerated aging with a progeria-like phenotype [52]. These mice die prematurely at about 3-4 weeks of age and are much smaller compared to their littermates [52]. When born, these mice are normal for about 2 weeks but they then exhibit severe metabolic defects. After 2 weeks, there is a decline in serum glucose levels causing a state of hypoglycemia and low plasma Igf1 levels – which is critical for bone

development. This results in brittle and thin bones and curved spine (termed lordokyphosis). These metabolic defects along with the premature aging phenotype with early death at 3 weeks of age make Sirt6 extremely interesting candidate for age-related metabolic disorders such as cardiovascular disease and diabetes mellitus.

At the **cellular level**, the absence of Sirt6 results in genomic instability and increased susceptibility to damage induced by ionizing radiation (IR), methylmethanesulfonate (MMS), and hydrogen peroxide (H_2O_2) [53]. Loss of Sirt6 leads to the formation of dysfunctional telomeres, accumulation of telomeric DNA damage foci, and genomic instability with chromosomal end-to-end fusions that drive the cell into premature senescence [54]. Sirt6 also plays a role in base excision repair (BER) and double-strand break (DSB) repair [55, 56]. Furthermore, Sirt6 also ADP-ribosylates PARP1 and stimulate its poly-ADP-ribosylation activity [57]. Since PARP1 is also involved in both BER and DSB repair, the activation of PARP1 by Sirt6 may also explain the BER defects observed in Sirt6-deficient cells. The defective BER, DSB repair and telomere-dependent genomic instability that might in part explain premature aging and cellular senescence seen in Sirt6 knockout mice.

Thus, the Sirt6 loss-of-function phenotypes at the cellular and whole organism levels imply that functional Sirt6 plays a protective role in many aspects of organismal health.

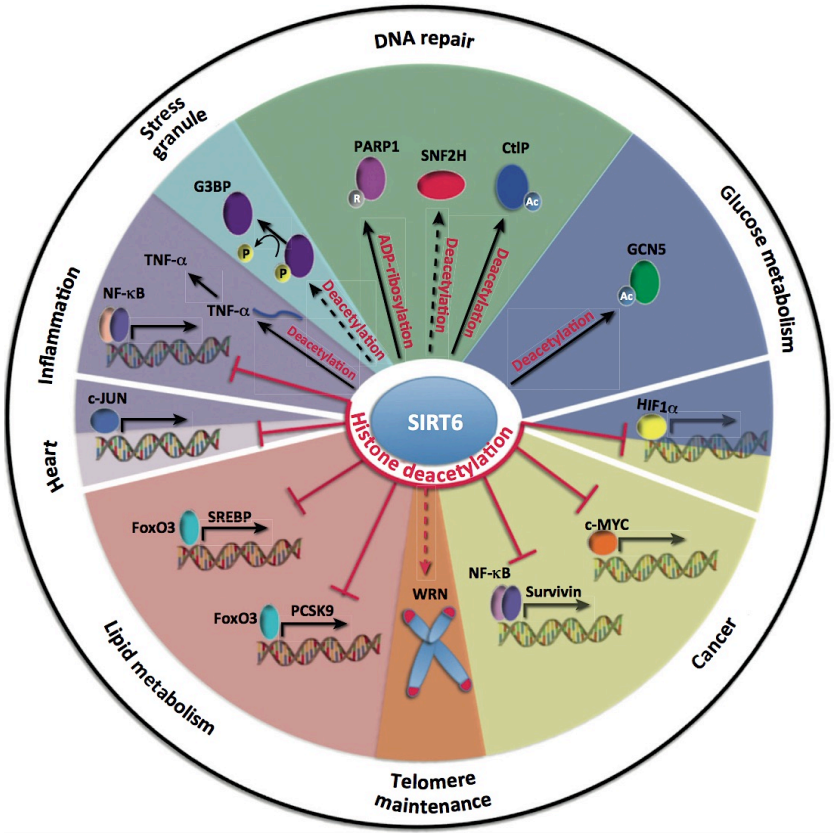

Figure 5: The cellular functions of Sirt6 and the impact on organismal biology and disease. Sirt6 primarily functions as a H3K9 and H3K56 histone deacetylase that decreases chromatin accessibility of transcription factors such as nuclear factor κB (NF-κB), c-JUN, Foxo3, MYC, and hypoxia inducible factor 1 α (HIF1α) to their respective target promoters, thus inhibiting target gene expression. Sirt6 can also directly regulate protein activity through deacetylation of GCN5 (general control non- repressed protein 5) and CtIP [C-terminal binding protein (CtBP) interacting protein] and ADP-ribosylation of poly- (ADP-ribose) polymerase 1 (PARP1). Sirt6 also has additional roles G3BP dephosphorylation and tumor necrosis factor-α (TNF-α) secretion by lysine deacylation (Adapted from Kugel et al., Trends in Biochemical Sciences, 2014) [58].

3.8 Role of Sirt6 in metabolism

Sirt6 is a key regulator of **glucose metabolism** (Figure 5). As previously described, Sirt6 knockout mice display hypoglycemic effects, which ultimately leads to their

death [52]. A study investigating whether Sir6 is directly or indirectly involved in glucose metabolism demonstrated that Sirt6-deficient embryonic stem cells have an upregulation of glycolytic genes such as *Ldh*, triose phosphate isomerase (*Tpi*), aldolase, and the rate- limiting glycolytic enzyme phosphofructokinase-1 (*Pfk-1*) [59]. There was also an increase in pyruvate dehydrogenase kinase genes *Pdk1* and *Pdk4*. These enzymes phosphorylate and inhibit PDH, the rate-limiting enzyme that regulates entrance of pyruvate into the tricarboxylic acid (TCA) cycle [59]. Sirt6 modulates glucose homeostasis specifically through a Hif1α -dependent mechanism. Sirt6 acts as a co-repressor of Hif1α. In the absence of Sirt6, the Hif1-α regulated glycolytic gene expression increases. Treatment of Sirt6-deficient mice with a HIF1α inhibitor rescued the hypoglycemic phenotype [60]. Additionally, in Sirt6-deficient cells there is an increase in the glucose transporter Glut1 and a pronounced increase in the uptake of glucose by muscles and the brown adipose tissue (BAT), both of which might also partially explain the hypoglycemic effect [59].

Sirt6 modulates **lipid metabolism** by influencing lipid clearance pathways (Figure 5). Hepatocyte-specific deletion of Sirt6 in mice causes an increase in liver steatosis in 90% of the mice from 7.5–13 months of age. These mice also have an increase in hepatic triglyceride levels due to an increase in triglyceride synthesis [61]. Livers of mice with hepatocyte-specific Sirt6 deletion show a higher expression of fatty acid translocase (*Fat*) with increased uptake of long chain fatty acids from blood [61].

Conversely, overexpression of Sirt6 improves lipid metabolism. Sirt6 overexpressing mice when fed a high-fat diet (HFD) were found to have decreased

visceral fat accumulation, improved blood lipid profile, glucose tolerance, and insulin secretion, and reduced expression of selected PPARγ-regulated genes involved in lipid metabolism, lipid transport, and adipogenesis [62]. Sirt6 overexpressing mice had lower levels of LDL-c, -one of the main protagonists in cardiovascular disease (60). One of the mechanisms by which Sirt6 lowers LDL-c is by regulating the expression of proprotein convertase subtilisin/kexin type 9 (Pcsk9). Secreted Pcsk9 is known to function as a chaperone and targets the LDLR for lysosomal degradation within hepatocytes [63]. FoxO3 recruits Sirt6 to the promoter of the *Pcsk9* gene where it deacetylates histone H3K9 and H3K56, to suppress Pcsk9 gene expression [64]. By lowering Pcsk9, Sirt6 improves LDLR recycling and increases clearance of LDL-c [64].

Sirt6 also modulates lipid metabolism by regulating the sterol-sensing transcription actor sterol-regulatory element binding protein (Srebp) (described in Figure 5). Srebp2, an isoform of Srebp, is a key regulator of cholesterol biosynthesis. More than one mechanism had been uncovered by means of which Sirt6 controls Srebp2 function. FoxO3 recruits Sirt6 to the Srebp2 gene promoter where it deacetylates H3K9 and H3K56 at Srebp promoter and downregulates the transcription of its target genes [65, 66]. Alternatively, Sirt6 also inhibits the cleavage of Srebp1/Srebp2 into their active forms by decreasing the transcription of the Srebp1/Srebp2 protease complexes (SCAP, S1P, and S2P) [66]. The third mechanism of modulation Srebp2 function is by the regulation of AMP/ATP ratio by Sirt6. This causes the activation of AMPK, which phosphorylates Srebp1 on Ser372, thus inactivating SREBP1 by suppressing its cleavage and nuclear translocation [66].

Sirt6 can also hydrolytically remove long-chain fatty-acyl groups, including myristoyl and palmitoyl groups, a process known as lysine deacylation [67]. Deacylation on lysine residues may occur on histones or other cellular proteins. Acyl-group removal would modify enzyme activity or chromatin regulation. Depending on the nutrient status, the cell may favor the addition of one acyl group over another. This is dependent on the levels of acyl-CoA, which can be formed as intermediates of various metabolic pathways such as fatty acid synthesis, beta oxidation, glycolysis, and the tricarboxylic acid (TCA) cycle. The many mechanisms by which Sirt6 regulates lipid metabolism highlight its importance for lipid homeostasis.

3.9 Sirt6 and inflammation

The NF-κB family of transcription factors is important in the inflammatory response that interacts with many metabolic and aging pathways. Numerous studies link sirtuins to NF-κB mediated signaling [68-70]. Sirt1 has been shown to deacetylate NF-κB and downregulates its activity [68, 69]. Recently, studies by Kawahara et al. demonstrated that Sirt6 interacts with RelA of the NF-κB subfamily and is recruited to the promoters of RelA target genes [70]. This in turn decreases the expression of NF-κB target genes through deacetylation of H3K9 at target gene promoters [70]. Along these lines, heterozygous deletion of RelA restores the reduced lifespan and aging- related phenotypes observed in Sirt6 KO mice [70].

Sirt6 homozygous KO mice on a mixed genetic background of 129/Black Swiss/FVB suffer from inflammation of the liver [71]. Sirt6 deficiency results in the

activation of c-Jun, and the downstream inflammatory target genes such as Il-6 and Mcp1, indicating that inflammatory responses of Sirt6 loss-of-function in macrophages activates c-Jun signaling [71].

Above examples highlight the anti-inflammatory aspects of Sirt6-mediated responses. However, Sirt6 can also exert an inflammatory response under certain conditions. For example, Sirt6 can catalyze the hydrolysis of myristoylated lysine 19 and 20 of tumor necrosis factor α (TNF-α), which enables the secretion of TNF-α from the mouse embryonic fibroblast (MEF) cells [67]. TNF-α is a key proinflammatory cytokine that is known to play a major part in numerous inflammatory diseases. *In vivo,* TNF-α has lower lysine fatty acylation and is more efficiently secreted from Sirt6 wild type macrophages compared to Sirt6 KO macrophages [67].

3.10 Sirt6 in cardiovascular disease

Sirt6 is known to play a role in cardiovascular disease. Sirt6 deficiency causes an increase in cardiac hypertrophy and degenerative changes to the heart. In line with this it has been shown that compared to healthy hearts, failing hearts have lower Sirt6 expression levels [72]. Furthermore, hearts from Sirt6-deficient mice have an increase in expression of cardiac fetal genes, an increase size of cardiomyocytes and an increase in interstitial fibrosis. While on the other hand, Sirt6 overexpression blocks these cardiac hypertrophic response. Mechanistically, Sirt6 interacts with c-JUN, which results in the blocking of IGF signaling by the deacetylation of H3K9 at

IGF downstream targets. Inhibition of c-JUN or IGF signaling blocks hypertrophy in Sirt6-deficient hearts [72].

Recent studies also show that Sirt6 may be protective in atherosclerosis. Compared to normal arteries, Sirt6 levels are decreased in atherosclerotic aortae of Apoe KO mice and in human plaques [73]. These findings suggest an atheroprotective role of Sirt6. However, the role of Sirt6 in macrophages, the mechanism and consequently its effect on atherosclerosis remains to be elucidated and thus are the objectives of this PhD thesis.

4. PROJECT HYPOTHESIS, AIMS AND EXPERIMENTAL DESIGN

4.1 Project Hypothesis

Given the involvement of Sirt6 in multiple aspects of lipid metabolism and anti-inflammatory responses and the relevance of these pathways in atherosclerosis, we hypothesized that

Sirt6 deficiency increases atherosclerosis by enhancing inflammation via activating NF-κB signalling and macrophage scavenger receptor 1 (Msr1) expression in macrophages.

4.2 Project Aims

To address above hypothesis, we pursued a step-wise approach with the following project aims:

<u>Aim 1</u>: To investigate the effects of partial deletion of *Sirt6* on atherosclerosis in *Apoe*$^{-/-}$ mice

For this purpose, we analysed the effects of heterozygous deletion of *Sirt6* on atherosclerosis, NFκB-mediated inflammation and LDL cholesterol metabolism in *Apoe*$^{-/-}$ mice.

<u>Aim 2</u>: To investigate the effects of homozygous deletion of *Sirt6* in bone marrow-derived cells in *Apoe*$^{-/-}$ mice.

To this end, we analyzed the effects of bone marrow transplantation of Sirt6-deficient bone marrow cells into *Apoe*$^{-/-}$ recipient mice on atherogenesis, foam cell formation and scavenger receptor expression.

To improve mechanistic insight, we used cultured RAW macrophages with genetic modulation of Sirt6 (knockdown and overexpression) – again investigating foam cell formation, lipid uptake and scavenger receptor expression.

4.3 Experimental design

<u>Project 1</u>: To study the effects of partial deletion of *Sirt6* on atherosclerosis

To test the effects of heterozygous deletion of Sirt6 on atherosclerosis, 8 weeks old male *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Sirt6*$^{+/-}$ mice were fed a high-cholesterol diet (HCD) with 1.25% cholesterol for 12 weeks (Figure 6). At the end of 12 weeks, the mice were harvested to assess for total *en face* thoracoabdominal plaque content by oil red O staining. Plasma total cholesterol, triglycerides and various lipoprotein sub fractions were measured. Other soluble parameters such as circulating cytokines were measured by ELISA.

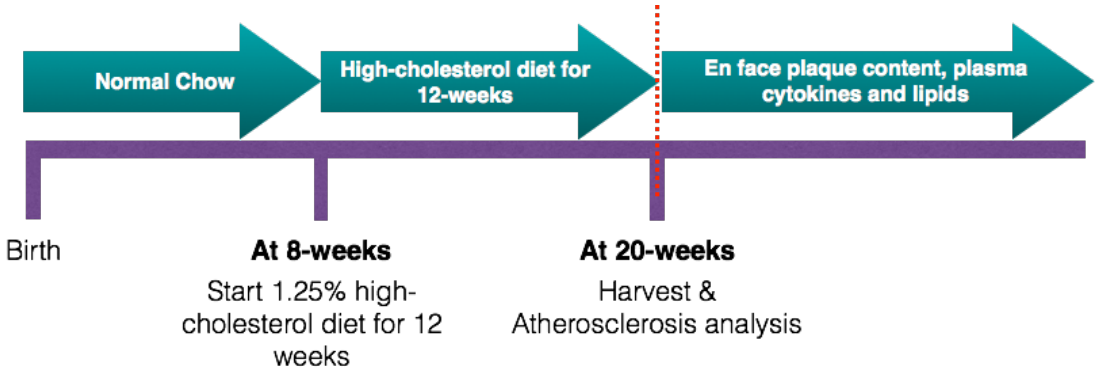

Figure 6: Experimental design for Sirt6 heterozygous deletion in Apoe KO mice. At 8 weeks of age, *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Sirt6*$^{+/-}$ mice were fed a high-cholesterol diet (HCD) for 12 weeks, followed by atherosclerosis analyses.

<u>Project 2</u>: To study the effect of bone marrow-specific deletion of Sirt6 on atherogenesis

To assess the effect of bone marrow-specific deletion of Sirt6 in Apoe KO mice on atherosclerosis first chimeric mice were generated. Donor bone marrow (BM) cells from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Sirt6*$^{-/-}$ mice were isolated. *Apoe*$^{-/-}$ recipient mice had been irradiated to destroy the resident BM cells and then injected with donor BM cells. The recipient *Apoe*$^{-/-}$ mice were then allowed 6 weeks for complete BM reconstitution after which they were fed a high-cholesterol diet (HCD) for 12 weeks (scheme as depicted in Figure 7), followed by atherosclerosis analyses as done for the Sirt6 heterozygous mice project.

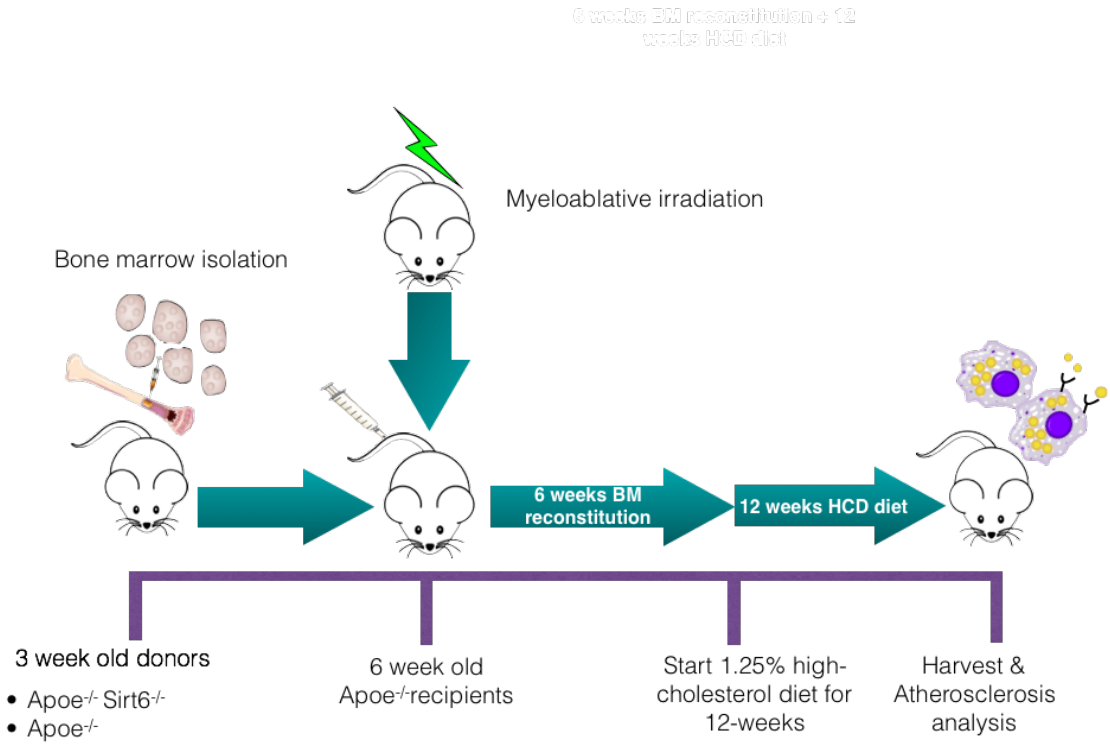

Figure 7: Experimental design for bone marrow-specific deletion of Sirt6 in Apoe KO mice: Bone marrow-specific Sirt6 depleted chimeric mice were generated by injecting *Apoe*$^{-/-}$ mice with Sirt6 deficient BM. These mice were then fed a high-cholesterol diet (HCD) for 12 weeks, followed by atherosclerosis analyses.

5. RESULTS

5.1 Primary publication (Summary)

Bone marrow-specific *Sirt6* enhances atherosclerosis by increased macrophage scavenger receptor 1 expression

Arsiwala T, van Tits LJ, Bisceglie L, Miranda M, Nussbaum K, Stivala S, Blyszczuk P, Weber J, Tailleux A, Stein S, Beer JH, Greter M, Becher B, Hottiger MO, Mostoslavsky R, Eriksson U, Staels B, Auwerx J, Lüscher TF, Matter CM

Contributions: Experimental design, planning and execution, manuscript writing and figures preparation.

Experiments performed for:

1. Figure 1. A-E
2. Figure 2. A-E, H-I
3. Figure 3. A-D
4. Figure 4. A-F
5. Figure 5. A-F

5.2 Primary Publication (prefinal version)

Bone marrow-specific *Sirt6* deletion enhances atherosclerosis by increasing macrophage scavenger receptor 1 expression

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ABSTRACT

Background: Macrophage Scavenger Receptor 1 (Msr1) mediates the uptake of modified low-density lipoproteins (mLDL) by macrophages, a critical step in atherogenesis. Sirt6 is a nuclear NAD^+-dependent deacetylase that is activated upon caloric restriction and provides protection in age-related diseases. The role of Sirt6 in atherogenesis is unknown.

Methods and Results: Heterozygous constitutive Sirt6 deletion did not affect atherosclerosis in *Apoe*$^{-/-}$ mice fed a high-cholesterol diet (HCD) for 12 weeks. However, bone marrow (BM)-specific *Sirt6* deletion increased aortic plaque formation in *Apoe*$^{-/-}$ mice on a HCD. Aortic root sections from *Sirt6*-deficient BM-transplanted mice revealed an increase in lipid content, macrophage numbers, Vcam1 expression and macrophage Msr1 levels. Plasma lipids and circulating cytokines were similar between the groups. In RAW 264.7 macrophages, Sirt6 knockdown enhanced Dil-labelled oxLDL uptake, whereas *Sirt6* overexpression reduced it; silencing of *Sirt6* increased mRNA and protein expression of Msr1, whereas the expression of other macrophage lipoprotein uptake and efflux receptors remained unchanged. Double knockdown of Sirt6 and Msr1 in RAW cells abolished the increase in oxLDL uptake. BM-derived macrophages revealed increased Msr1 protein expression in *Apoe*$^{-/-}$ *Sirt6*$^{-/-}$ compared with *Apoe*$^{-/-}$ *Sirt6*$^{+/+}$ donors. Flow cytometric analysis of macrophages from aortic plaques of *Sirt6*-deficient BM-transplanted mice showed increased Msr1 expression; similar results were found in BM cells; however, analyses in blood monocytes showed no difference in Msr1 expression.

Conclusions: Loss of *Sirt6* in BM-derived macrophages is proatherogenic through an increase in Msr1 expression and enhanced uptake of oxLDL by macrophages. These findings suggest a macrophage-specific atheroprotective role of Sirt6.

INTRODUCTION

Atherosclerosis is the leading cause of death in developed countries. Excess plasma low-density lipoproteins (LDL) are a key driver of atherosclerosis. Excess LDL is retained in the subendothelial space. If exposed to oxidative stress, they may transform into oxidised LDL (oxLDL). This modified LDL (mLDL) is taken up by scavenger receptors such as scavenger receptor A, synonymous in mice with mouse scavenger receptor 1 (Msr1), CD36 and oxidized low-density lipoprotein receptor 1 (Lox1) leading to macrophage foam cell formation. Atherogenesis occurs when these excess circulating LDL-cholesterol (LDL-c) and its modified forms accumulate within the vascular intima triggering endothelial dysfunction, local inflammation, plaque formation and vascular remodelling leading to the narrowing of coronary or cerebral arteries which may eventually culminate in myocardial infarction or stroke, respectively.

Sirtuins are a family of NAD^+ dependent deacetylases that are activated upon caloric restriction and prevent age-associated diseases. Sirt6 is a nuclear, chromatin-associated protein and is known to deacetylate histone H3 lysine 9 (H3K9) [74] and H3 lysine 56 (H3K56) [75]. By means of this histone deacetylation, Sirt6 maintains chromatin conformation and regulates telomere stability, DNA repair and gene expression [74]. Loss-of-function studies reveal that Sirt6 plays a key role in aging, metabolic homeostasis and inflammation control: Sirt6-null mice are small for their age, develop accelerated aging and severe hypoglycemia with premature mortality at around 4 weeks of age [76]. Interestingly, Sirt6-null mice surviving early death by feeding water with 10% glucose and mice with liver-specific deletion of Sirt6 develop chronic liver inflammation and fibrosis, in part due to c-Jun activation [77, 78]. Similarly, bone marrow-derived macrophages isolated from Sirt6-null mice show increased Mcp-1, Il-6, and TNFα expression levels and are hypersensitive to LPS stimulation [77].

Sirt6 has been shown to have an important role in metabolic diseases: Sirt6 heterozygous mice on an S129v background confer an increased risk of metabolic syndrome with enhanced inflammation [79]. Conversely, constitutive overexpression

of Sirt6 protects from metabolic disturbances induced by a high-fat diet [62]. However, the effects of homozygous Sirt6 deletion in macrophages on atherogenesis and foam cell formation remain unknown.

Thus, we hypothesized that partial constitutive and/or bone-marrow-specific Sirt*6* deletion in *Apoe*$^{-/-}$ mice increases atherogenesis by its pro-inflammatory actions on macrophage foam cell formation. To test this hypothesis, we performed genetic Sirt6 loss-of-function experiments in cultured macrophages using a knockdown approach and in atherosclerotic apolipoprotein E knockout (*Apoe*$^{-/-}$) mice using constitutive heterozygous or bone marrow-specific Sirt6 deletion.

MATERIALS AND METHODS

Animals

Apoe$^{-/-}$ *Sirt6*$^{+/-}$ mice (provided by Johan Auwerx, EPFL) and *Apoe*$^{-/-}$ (Charles River, Italy) were bred in-house under pathogen–free conditions housed with a 12 hours light – dark cycle and fed a high-cholesterol diet (HCD) containing 1.25% cholesterol (D12108; Research Diets, New Brunswick, USA) for 12 weeks. All experiments and animal care procedures were approved by the local and cantonal veterinary authorities and carried out in accordance with the local institutional guidelines.

For the generation of bone marrow chimeras, 6 week-old female *Apoe*$^{-/-}$ recipient mice were sub-lethally irradiated with two doses of 550 rads each. 3 week-old donor mice were killed by CO_2 inhalation and bones (hind limbs and hips) were flushed with sterile PBS to obtain bone marrow (BM) cells. Donor BM cells were injected intravenously (2×10^7 total cells per mouse), and to prevent bacterial infection 0.2% (vol/vol) Borgal (trimethoprim, sulfadoxin) was added to the drinking water for 1 week and mice were allowed to recover for 6 weeks and then were exposed to 12-weeks of HCD. To confirm bone marrow chimerism, a control experiment with CD45.1 donor BM cells injected into CD45.2 positive *Apoe*$^{-/-}$ mice was performed: Cell mixtures were assessed by flow cytometry with monoclonal antibody to CD45.1 (A20; BD Pharmingen) and monoclonal antibody to CD45.2 (104; BD Pharmingen).

Cell culture

RAW 264.7 macrophages (Sigma-Aldrich) were cultured in a 5% CO_2, 3% O_2 incubator at 37 °C in DMEM-high glucose medium supplemented with 2mM Glutamine and 10% FBS (v/v). In order to obtain BM-derived macrophages (BMDM), BM cells were differentiated into macrophages using 10ng/ml M-CSF for one week.

Transfections

Knockdown experiments were performed using lipofectamine RNAiMAX (Life Technologies). For the adenovirus transduction experiments, control and Sirt6 adenovirus was obtained from SignaGen.

In vitro lipid uptake

24 hours following protein knockdown, RAW 264.7 cells were treated with 50µg/ml of Dil-labelled oxLDL (Kalen Biomedical). After treatment with Dil-oxLDL for 24 hours the uptake was measured by flow cytometry.

Immunohistochemistry, and Immunofluorescence Analyses

Aortic root tissues were embedded in OCT and sectioned (5µm thick). For immunohistochemistry and immunofluorescence, the sections were fixed in acetone. Antibodies against Vcam1 and Cd68 were from AbD Serotec; Sirt6 was from Cell Signaling Technologies. For IF, primary anti- body reaction, sections were stained with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) (Sirt6) or Alexa Fluor 547 goat anti- Rat IgG (eBioscience) (Cd68) for 1 h.

Cytokine Assays

Inflammatory cytokines (Mcp-1 and Il-6) were measured using ELISA kits from Thermo Scientific according to the manufacturer's protocol.

Cholesterol profiles and lipoprotein fractions

Cholesterol and triglyceride plasma concentrations were measured using colorimetric and enzymatic methods with ready to use kits (BioMérieux). To assess the distribution of cholesterol and triglycerides over the different lipoprotein fractions, lipoproteins were separated by size exclusion chromatography followed by online determination of lipids.[80]

Flow Cytometry

Flow cytometry analyses were performed using an LSRII Fortessa (Becton Dickinson) and analysed with FlowJo software (Tree Star). Cells isolated from the blood and aortic arch were stained with specific fluorochrome-conjugated – CD45, F4/80, CE11b, propidium iodide (Ebioscience), Msr1 (Abcam).

Western blotting and quantitative RT-PCR

Western blot analysis was carried out according to standard procedures using ECL detection from Millipore (Billerica, MA). The following antibodies were used: anti-β actin (Cell Signaling Technology), anti- Sirt6, anti-acH3K9 (Cell Signaling Technology), and anti-acH3K56 (Abcam). For quantitative PCR, the total RNA was extracted from tissues and cells using TRIzol reagent (Life technologies) and reverse-transcribed into cDNA using MML-V reverse transcriptase or Superscript II RT, followed by PCR amplification with Platinum SYBR Green qPCR SuperMix-UDG (all from Invitrogen). The following primers were used:

Gene	**Forward (5'-3')**	**Reverse (5'-3')**
Sirt6	ATGTCGGTGAATTATGCAGCA	GCTGGAGGACTGCCACATTA
Cd36	ATGGGCTGTGATCGGAACTG	GTCTTCCCAATAAGCATGTCTCC
Lox1	CAAGATGAAGCCTGCGAATGA	ACCTGGCGTAATTGTGTCCAC
Marco	ACAGAGCCGATTTTGACCAAG	CAGCAGTGCAGTACCTGCC
Msr1	GCACAATCTGTGATGATCGCT	CCCAGCATCTTCTGAATGTGAA
Scarb1	AAACAGGGAAGATCGAGCCAG	GGTCTGACCAAGCTATCAGGTT
Scarb2	ATGAGTGTGGGATAGCGGTGT	GGCACTTTGTGTCGCAGTT
Ldlr	AGTGGCCCCGAATCATTGAC	CATGCACAAGGTCCTGAGAA
Abca1	CTAACTAAACACCAGACAGAGGC	CATGCACAAGGTCCTGAGAA
Abcg1	CTTTCCTACTCTGTACCCGAGG	CGGGGCATTCCATTGATAAGG

Statistics

All data are presented as mean ± SEM. Data distribution was assessed using the Kolmogorov – Smirnov test. Normally distributed data were compared using an unpaired two-tailed Student's t-test; for non-parametric data the Mann – Whitney test was used. Three or more groups were compared using a Kruskal – Wallis test followed by a Dunn's post-hoc comparison (non-parametric data). At least three independent experiments in triplicates were performed. Significance was accepted at $p \leq 0.05$. Analyses were performed using Graphpad Prism (version 5.0d, 2010).

RESULTS

Heterozygous constitutive deletion of *Sirt6* does not affect atherosclerosis in *Apoe*$^{-/-}$ mice

Since constitutive homozygous Sirt6 deletion confers embryonic and early life lethality [76], we assessed the effects of constitutive heterozygous *Sirt6* deletion on atherosclerosis. Thus, *Apoe*$^{-/-}$*Sirt6*$^{+/-}$ *and Apoe*$^{-/-}$*Sirt6*$^{+/+}$ mice were fed a HCD for 12 weeks. Comparisons of thoraco-abdominal aortae e*n face* and serial aortic root cross sections by oil-red O staining showed no difference between these in total plaque area and lipid deposition, respectively (**Figure 1A, B**). Immunohistochemical analyses revealed no differences in Vcam1 and Cd68 expression in aortic root cross sections from *Apoe*$^{-/-}$*Sirt6*$^{+/-}$ mice compared to *Apoe*$^{-/-}$*Sirt6*$^{+/+}$ controls (**Figure 1C, D**).

Analyses of liver lysates revealed that *Apoe*$^{-/-}$*Sirt6*$^{+/-}$ mice expressed about 40% lower Sirt6 protein levels than *Apoe*$^{-/-}$*Sirt6*$^{+/+}$*;* however, there was no change in the acetylation status of the Sirt6 targets-H3K9 or H3K56 (**Figure 1E**). Plasma lipid analyses showed that cholesterol and triglyceride levels including lipoprotein subfractions were similar between the two groups (**Figure F-GH**). These studies indicate that constitutive heterozygous Sirt6 deletion in *Apoe*$^{-/-}$ mice is not sufficient to affect atherosclerosis.

Sirt6 deficiency in hematopoietic cells increases plaque formation in *Apoe*$^{-/-}$ mice

To test the effects of homozygous BM-specific Sirt6 deletion on atherosclerosis, BM from *Apoe*$^{-/-}$*Sirt6*$^{+/+}$ *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$*Sirt6*$^{-/-}$ donor mice were transplanted into irradiated *Apoe*$^{-/-}$ recipient mice. Following 6 weeks of recovery with BM reconstitution, mice were fed a HCD for 12 weeks. *En face* plaque staining of the thoraco-abdominal aortae revealed a significant increase in atherosclerosis in *Apoe*$^{-/-}$ *Sirt6*$^{-/-}$ BM-recipient mice compared with *Apoe*$^{-/-}$*Sirt6*$^{+/+}$ *Apoe*$^{-/-}$ BM-recipient mice (**Figure 2A**). Blood haematological analyses showed that the count of the cell types is similar between both the groups after BM-transplantation (Supplemental Table 1). Oil-red O staining of serial aortic root cross sections showed an increase in lipid accumulation and lesion size in *Apoe*$^{-/-}$*Sirt6*$^{-/-}$ BM-recipient mice (**Figure 2B**)

compared with *Apoe*$^{-/-}$*Sirt6*$^{+/+}$ *Apoe*$^{-/-}$ BM-recipient control mice. Furthermore, aortic root cross-sections from the *Apoe*$^{-/-}$*Sirt6*$^{-/-}$ BM-recipient mice showed increased endothelial activation (Vcam1- positive) and macrophages (Cd68-positive staining) within plaques (**Figure 2C, D**). BM cells isolated from *Apoe*$^{-/-}$*Sirt6*$^{-/-}$ BM recipient mice confirmed the loss of Sirt6 and its deacetylase activity showing a lack of Sirt6 protein expression as well as an increase in H3K9 and, H3K56 acetylation levels, respectively (**Figure 2E**). Plasma lipid analyses showed that cholesterol and its lipoprotein subfractions were similar between the two groups (**Figure 2F-G**) and additionally, no difference was measured in the soluble plasma cytokines like Mcp-1 and IL-6 between both the groups (**Figure 2H**). Co-staining for the macrophages in the aortic plaque and Sirt6 revealed there was no Sirt6 present in the macrophages in *Apoe*$^{-/-}$ *Sirt6*$^{-/-}$ BM recipient mice confirming the accumulated plaque macrophages are BM-derived cells (Supplemental Figure 1A).

Sirt6 regulates macrophage oxLDL accumulation

To determine whether macrophage-specific deficiency of Sirt6 influences lipoprotein uptake, *in vitro* in RAW 264.7 cell accumulation of oxLDL was measured. First, we confirmed that the expression levels of Sirt6 are reduced upon Sirt6 knockdown (**Figure 3A**). *Sirt6* silencing increased oxLDL accumulation *in vitro* (**Figure 3B, C**). Conversely, Sirt6 overexpression (**Figure 3D**) reduced oxLDL accumulation (**Figure 3E, F**). These findings indicate that macrophages with the deficiency of Sirt6 are more prone to foam cell formation upon exposure to mLDL.

Sirt6 limits macrophage oxLDL accumulation by suppressing Msr1 expression

To address the mechanism underlying the increase in oxLDL uptake upon Sirt6 deficiency, we assessed the effects of silencing of Sirt6 on the expression of genes involved in cellular cholesterol accumulation. Knockdown of Sirt6 increased scavenger receptor Msr1 mRNA and protein expression, but did not alter mRNA expression of other scavenger receptors such as *Cd36* or *Lox1*. (**Figure 4A, B**). Furthermore, mRNA levels of the cholesterol efflux transporters *Abca1* and *Abcg1* were not altered (**Figure 4A**). Conversely, adenovirus-mediated overexpression of

Sirt6 reduced Msr1 protein expression compared with control adenovirus (**Figure 4C**). These data suggest that Sirt6 knockdown increased Msr1 expression and oxLDL accumulation, but they do not address whether Msr1 is sufficient to increase foam cell formation upon Sirt6 deletion.

For this purpose, Dil-oxLDL uptake was measured upon double knockdown of Msr1 and Sirt6. Msr1 knockdown abolished the increase in Dil-oxLDL uptake upon Sirt6 knockdown (**Figure 4D, E**), confirming that Msr1 is a necessary mediator for increased oxLDL accumulation in Sirt6-deficient macrophages. In parallel, Msr1 levels were increased in BMDM from *Apoe*$^{-/-}$*Sirt6*$^{-/-}$ compared with *Apoe*$^{-/-}$*Sirt6*$^{+/+}$ mice (**Figure 4F**) *ex vivo*.

Sirt6$^{-/-}$ BM transplantation increases Msr1 expression in BM cells and in mouse aortic plaque macrophages

To assess the *in vivo* relevance of Msr1 expression in our Sirt6-BM atherosclerosis phenotype, we analysed Msr1 expression in BM-recipient mice to ascertain whether absence of Sirt6 regulates Msr1 expression *in vivo*. BM cells isolated from *Apoe*$^{-/-}$ *Sirt6*$^{-/-}$ BM recipient mice after 12 weeks of HCD showed both increased mRNA and protein levels of Msr1 compared with BM cells from *Apoe*$^{-/-}$*Sirt6*$^{+/+}$ BM transplanted mice (**Figure 5A, B**). Immunofluorescence analyses of aortic root sections revealed increased Msr1 expression in macrophages of *Apoe*$^{-/-}$*Sirt6*$^{-/-}$ BM-recipient mice compared with *Apoe*$^{-/-}$*Sirt6*$^{+/+}$ BM-recipient mice (**Figure 5C**).

Furthermore, we assessed Msr1 levels in aortic arch plaque macrophages. Flow cytometric analyses showed an increased expression of Msr1 in the Ly6C^{+}F4/80^{high} aortic macrophages of *Apoe*$^{-/-}$*Sirt6*$^{-/-}$ BM-recipient mice relative to the *Apoe*$^{-/-}$ BM-recipient mice (**Figure 5D**). Interestingly, corresponding analyses in blood monocytes isolated from these mice showed no difference in Msr1 expression between both groups (**Figure 5E, F**). These findings suggest that Sirt6 regulates Msr1 particularly in macrophages and that this effect on aortic macrophages is lesion-specific.

Figure 1

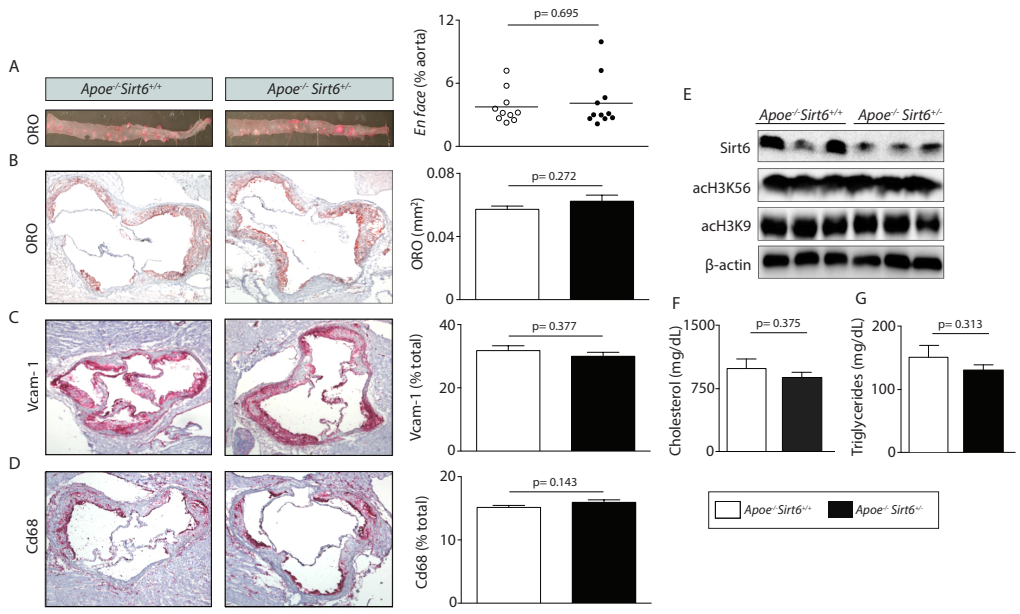


Figure 1. Constitutive heterozygous *Sirt6* deletion does not affect atherosclerosis in *Apoe*$^{-/-}$ mice. *Apoe*$^{-/-}$ *Sirt6*$^{+/-}$ *and Apoe*$^{-/-}$ *Sirt6*$^{+/+}$ mice were fed a high-cholesterol diet (HCD) for 12 weeks. **A,** Representative images of *en face* thoraco-abdominal aortae stained with Oil Red O (ORO, left panel) and corresponding plaque quantifications (right panel). **B**, Representative images and quantifications of aortic root cross sections stained with ORO. **C**, anti-Vcam1 **D**, anti-Cd68 (A-D, n=11). **E**, Sirt6, acetylated H3K56 and acetylated H3K9 protein expression in liver lysates of *Apoe*$^{-/-}$ *Sirt6*$^{+/+}$ and *Apoe*$^{-/-}$*Sirt6*$^{+/-}$ mice (n=3). **F,** Total plasma cholesterol levels. **G,** Plasma triglyceride concentrations.

Figure 2

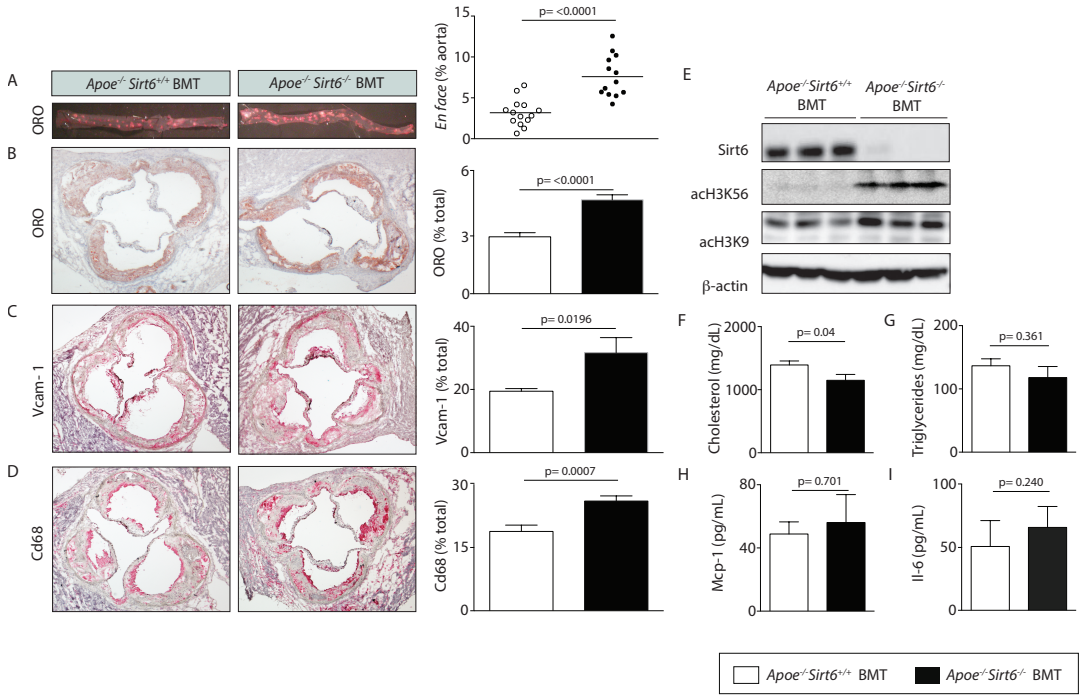


Figure 2. Hematopoietic homozygous *Sirt6* deficiency increases atherosclerosis. Bone marrow (BM) cells from *Apoe*$^{-/-}$ *Sirt6*$^{+/+}$ or *Apoe*$^{-/-}$ *Sirt6*$^{-/-}$ mice were transplanted into recipient *Apoe*$^{-/-}$ mice and fed a HCD diet for 12 weeks. **A,** Representative images of *en face* thoraco-abdominal aortae stained with Oil Red O (ORO, left panel) and corresponding plaque quantifications (n=13, right panel). **B-D**, Representative images (left panel) and quantifications of aortic root cross sections (right panel) stained for lipids (ORO), Vcam-1 and Cd68 (n=13). **E,** Western blot of Sirt6 and AcH3K56 expression levels in the BM lysates from the *Apoe*$^{-/-}$ *Sirt6*$^{+/+}$ or *Apoe*$^{-/-}$ *Sirt6*$^{-/-}$BMT mice. **F,** Total plasma cholesterol levels. **G,** Plasma triglyceride concentrations. **H,** Plasma levels of Mcp-1 and Il-6.

Figure 3

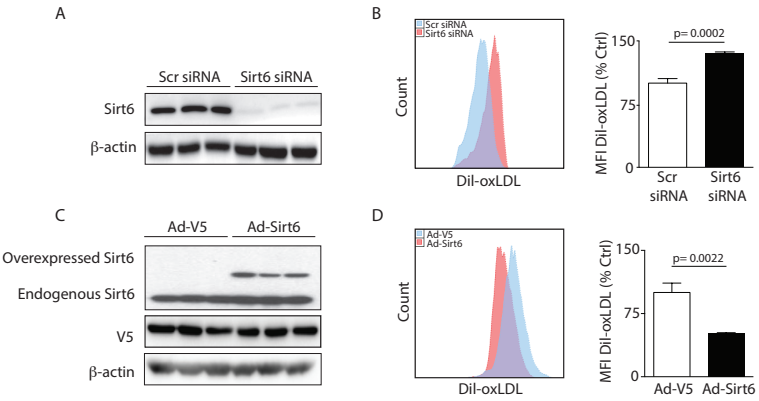


Figure 3. *Sirt6* downregulates macrophage oxLDL uptake in RAW macrophages. A, Western blot of Sirt6 protein upon scramble and Sirt6 knockdown. **B,** Representative histogram and quantification of Dil-oxLDL uptake upon scramble or Sirt6 knockdown. **C**, Western blot of Sirt6 protein levels upon the overexpression of Sirt6 using V5-tagged Sirt6 adenovirus (Ad-Sirt6) compared to control V5-adenovirus (Ad-V5). **D**, Representative histogram and quantification of Dil-oxLDL uptake upon Sirt6 overexpression.

Figure 4

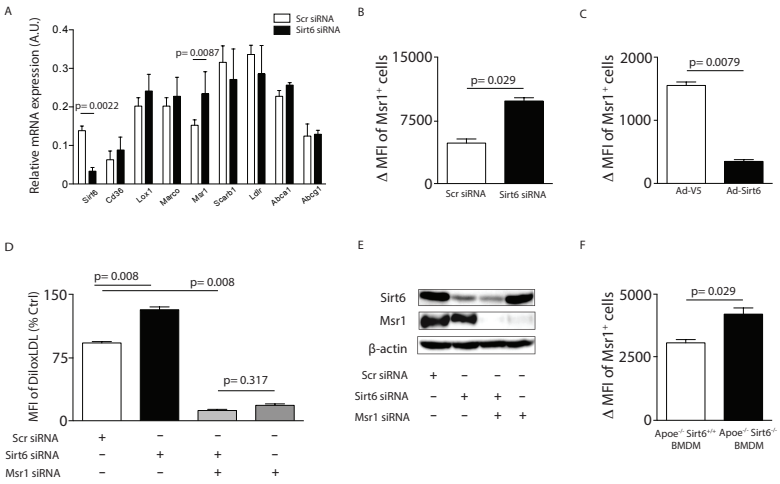


Figure 4. Sirt6 regulates macrophage oxLDL accumulation via Msr1. A, Relative mRNA expression levels of genes involved in modified low-density lipoprotein uptake and efflux. **B**, Knockdown of Sirt6 increases Msr1 protein compared to control levels in RAW 264.7 macrophages. **C**, Sirt6 overexpression decreases Msr1 protein levels compared to control adenovirus treatment in RAW 264.7 macrophages. **D**, Quantification of Dil-oxLDL upon knockdown of Sirt6 and Msr1. **E**, Protein expression of Sirt6 and Msr1 on control, single or double knockdown. **F**, Msr1 protein expression in BM-derived macrophages obtained from *Apoe*$^{-/-}$ *Sirt6*$^{-/-}$ and *Apoe*$^{-/-}$ mice.

Figure 5

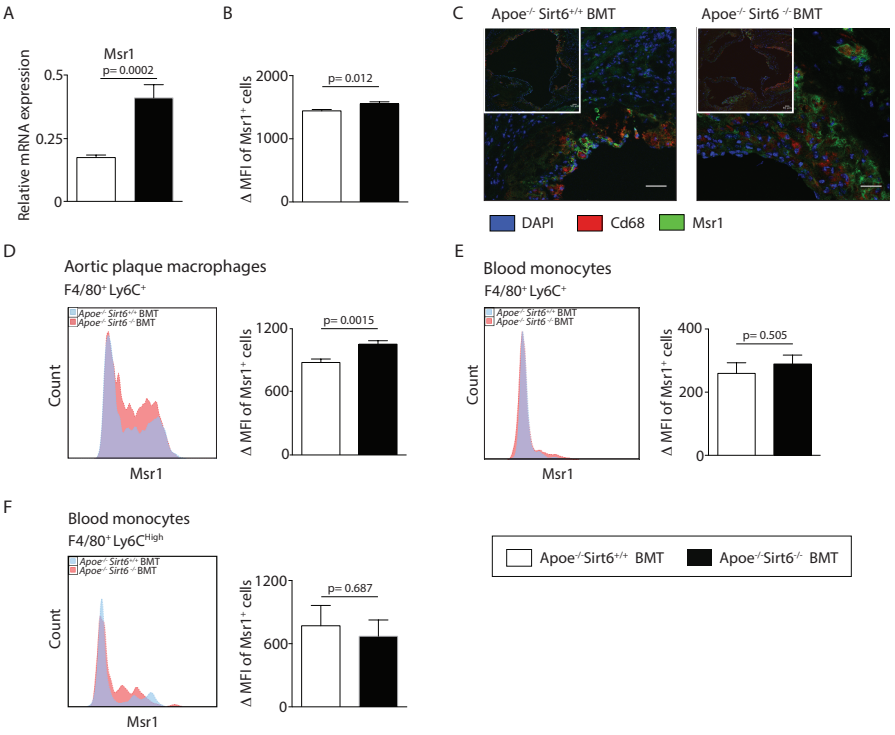
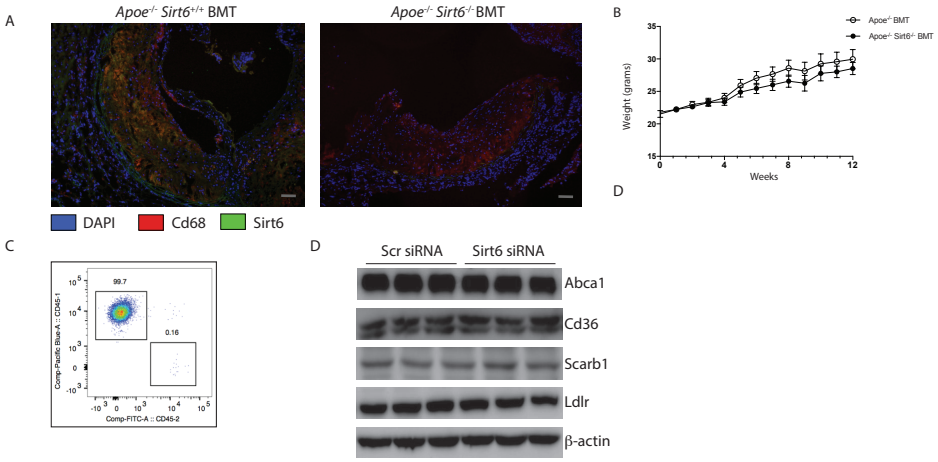


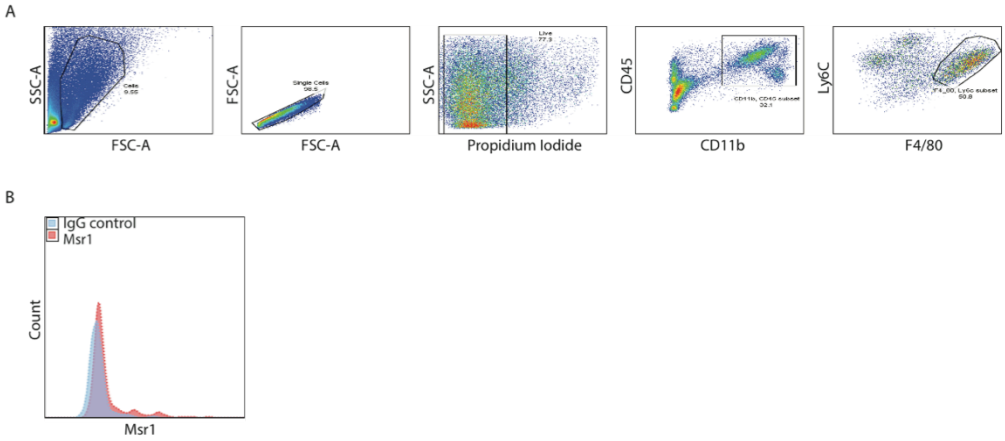
Figure 5. Hematopoietic *Sirt6* deficiency increases Msr1 expression in BM and aortic plaque macrophages. A, Msr1 mRNA and **B,** Msr1 protein expression is increased in the BM cells of *Apoe*$^{-/-}$ *Sirt6*$^{+/+}$ and *Apoe*$^{-/-}$*Sirt6*$^{-/-}$BM transplanted mice. **C**, Immunofluorescence of Msr1 and CD68 showing more macrophage-associated Msr1 expression in cross sections of the aortic root of *Apoe*$^{-/-}$ *Sirt6*$^{-/-}$BM transplanted mice compared with the *Apoe*$^{-/-}$ *Sirt6*$^{+/+}$ BM transplanted mice (bar = 20 mm, insert: bar = 30 mm). **D-E,** Msr1 expression levels are higher in Ly6C^{+}F4/80^{high} macrophage population in *Apoe*$^{-/-}$ *Sirt6*$^{-/-}$ BM transplanted mice compared with the *Apoe*$^{-/-}$ *Sirt6*$^{+/+}$ BM transplanted mice (n=7). **F-G**, Msr1 expression levels are unchanged in the F4/80^{+} Ly6C^{low} and F4/80^{+} Ly6C^{high} blood monocyte populations of *Apoe*$^{-/-}$ *Sirt6*$^{-/-}$and *Apoe*$^{-/-}$ *Sirt6*$^{+/+}$ BM transplanted mice.

Supplemental Figure 1



Supplemental Figure 1. A, Weight-course of *Apoe*$^{-/-}$ *Sirt6*$^{+/+}$ and *Apoe*$^{-/-}$*Sirt6*$^{-/-}$BM transplanted mice during 12-weeks on HCD. **B**, Sirt6 expression in aortic root sections showing the accumulated macrophages in *Apoe*$^{-/-}$*Sirt6*$^{-/-}$BM transplanted mice expresses no Sirt6. **C**, Control chimerism experiment using CD45.1 donor cells injected in CD45.2 *Apoe*$^{-/-}$ mice showing complete reconstitution of mice with donor BM. **D**, Protein expression of cholesterol efflux transporter- ABCA1 and scavenger receptors-Scarb1 and CD36 and Ldlr upon scramble and Sirt6 KD in RAW macrophages

Supplemental Figure 2



Supplemental Figure 2. A and B, Gating strategy to identify macrophages isolated from aortic root plaque cells and the corresponding histogram of MSr1 positive cells compared to IgG control.

Supplemental Table 1: Blood haematology counts from *Apoe*$^{-/-}$ *Sirt6*$^{+/+}$ and *Apoe*$^{-/-}$ *Sirt6*$^{-/-}$ BM transplanted mice.

BMT group	WBC (K/µl)	RBC (10^6/mm^3)	HGB (g/dl)	HCT (%)	MCV (fL)	PLT (10^3/mm^3)	Blood glucose (mmol/L)
Apoe$^{-/-}$ *Sirt6*$^{+/+}$ BMT	6.94	6.94	12.92	38.54	43.8	1070.8	10.38
Apoe$^{-/-}$ *Sirt6*$^{-/-}$ BMT	6.18	6.18	12	33.66	44.2	969.6	10.6
p-values	0.3413	0.4127	0.1508	0.0556	0.7063	0.3095	0.9524

DISCUSSION

As the nuclear Sirt6 exhibits a profound phenotype with a progeria-like syndrome with severe hypoglycemia and early lethality [52], the effects of constitutive homozygous Sirt6 deletion cannot be studied in atherosclerosis. Thus, we studied the effects of heterozygous deletion of Sirt6 in atherosclerotic *Apoe*$^{-/-}$ mice.

Patients with diabetes show lower Sirt6 expression in carotid atherosclerotic plaques than non-diabetic patients, suggesting that the risk factor diabetes is associated with low Sirt6 expression in human carotid atherosclerosis [81]. Recent studies have shown that Sirt6 heterozygous mice exhibit impaired endothelial dysfunction and increased oxidative stress [54]. As endothelial dysfunction precedes atherosclerosis, these reports imply that heterozygous *Sirt6* deletion would increase atherosclerosis. However, in our hands, using heterozygous constitutive *Sirt6* deletion was not sufficient to change atherosclerosis in *Apoe*$^{-/-}$ mice. We show that the heterozygous deletion of Sirt6 causes a 40% reduction of Sirt6 expression to control in the liver, however it is possible that this 40% reduction is not detrimental enough to elicit a phenotype. As complete loss of Sirt6 in mice is lethal after 4 weeks of age with severe metabolic defects, it is possible that the available Sirt6 in heterozygous Sirt6 knockout mice is already adequate for the body to prevent the adverse effects of the complete absence of Sirt6. Another possible explanation for the absence of an atherosclerosis phenotype is that the loss of single *Sirt6* allele triggers some compensatory effects that could offset the effects of less Sirt6.

These results are not consistent with published literature of heterozygous deletion of Sirt6 in atherosclerosis, in which partial deletion of Sirt6 is proatherosclerotic [82, 83]. This discrepancy might be a result of differences in experimental protocols. In one of the publications, the mice were fed a HFD instead of HCD for of 16 weeks starting at 4 weeks of age [83] instead of the 12-weeks on HCD we have used. While another paper fed the heterozygous Sirt6 knockout mice a HFD for duration of 8-weeks and then assessed for atherosclerosis [73].

As partial constitutive Sirt6 deletion was is not sufficient to affect

atherosclerosis, we selected macrophages as protagonist cell of atherosclerosis and tested the effects of complete Sirt6 deletion on atherogenesis. The bone-marrow-specific deletion of Sirt6 in *Apoe*$^{-/-}$ mice increased atherosclerosis, aortic macrophage accumulation and endothelial activation. Alternatively, BMDM from Sirt6 knockout mice *ex vivo* have increased Mcp-1, Il-6 and TNF-a expression levels [71] , however, we did not find a difference in plasma Mcp-1 and Il-6 levels between *Apoe*$^{-/-}$ *Sirt6*$^{-/-}$ and *Apoe*$^{-/-}$*Sirt6*$^{+/+}$ BM-recipient mice, suggesting that BM-restricted deletion of Sirt6 does not mediate its effects on atherosclerosis by increasing systemic inflammation. The clear atherosclerotic phenotype of Sirt6 matches the atherosclerotic phenotype of Sirt1 where the Sirt1 deletion in BM-derived cells was sufficient to increased atherosclerosis in *Apoe*$^{-/-}$ mice [84].

No reports exist about the role of **Sirt6 in modulating scavenger receptors**. We found that homozygous Sirt6 deletion in BM-derived cells increased both Msr1 mRNA and protein expression in *Apoe*$^{-/-}$ mice. Previous studies have shown that genetic Msr1 loss-of-function in *Apoe*$^{-/-}$ mice results in a 60% decrease in atherosclerotic lesions, highlighting the significance of Msr1 for the development of atherosclerosis in mice [85-88]. The increase in Msr1 expression upon Sirt6 deletion in both BM-derived macrophages (BMDM) from the donor BM cells before BM-transplantation and in BM-derived cells after BM-transplantation after 12 weeks of HCD suggests that Msr1 plays an important role for plaque formation in our atherosclerosis model.

To improve mechanistic insight, we extended our *in vivo* Sirt6 loss-of-function findings to an ***in vitro*** **model:** Sirt6 knockdown and overexpression studies in RAW 264.7 macrophages specified that Sirt6 knockdown indeed increased Msr1 mRNA and protein expression; the fact that Sirt6 deletion increased foam cell formation and oxLDL uptake gave these molecular changes a functional relevance and provided an explanation for the *in vivo* phenotype. Finally, the absence of oxLDL uptake upon double knockdown of Sirt6 and Msr1 confirmed that the increase in foam cell formation upon Sirt6 knockdown was Msr1-dependent and that Msr1 silencing was sufficient to abolish Sirt6-dependent oxLDL uptake.

Recent studies have suggested a rapid turnover of macrophages in atherosclerotic lesions. This increase in proliferation has been suggested to be due to an increase in Msr1 expression leading to an increase in atherosclerosis progression [89]. We identified $CD45^{+}CD11b^{+}$ $Ly6C^{+}$ F4/80 ^{high} macrophages by flow cytometry from plaques of aortic arches of *Apoe*$^{-/-}$*Sirt6*$^{-/-}$ and *Apoe*$^{-/-}$*Sirt6*$^{+/+}$ BM recipient mice fed a HCD. We found a significant increase in Msr1 expression in the macrophage populations isolated from the aortic arch of *Apoe*$^{-/-}$*Sirt6*$^{-/-}$ BMT mice compared to *Apoe*$^{-/-}$*Sirt6*$^{+/+}$ BMT mice. Conversely, blood monocytes isolated from the same mice showed no difference in Msr1 expression. This suggests that effect of Sirt6 on Msr1 expression is specific to macrophages and occurs in response to the increased lipids or local inflammatory mediators in the arch lesion site. Another explanation for the increased Msr1 on macrophages from *Apoe*$^{-/-}$*Sirt6*$^{-/-}$ BMT mice is that the expression of Msr1 is dependent on the maturity of the cells and the degree of differentiation. It might be that the differentiation of the monocytes to form the monocyte-derived macrophages regulates the expression of Msr1 on the macrophages as is the case for several other antigens [90].

CONCLUSIONS AND PERSPECTIVES

The accumulation of modified forms of LDL within the aortic vessel is a hallmark of atherosclerosis and contributes to an increased risk of coronary events. Here, we demonstrated that the deficiency of Sirt6 in macrophages is proatherosclerotic and upregulates Msr1 expression both *in vivo* and *in vitro*. In RAW macrophages Sirt6 is able to downregulate Msr1 expression and prevent the increased oxLDL accumulation. As it has been reported that Sirt6 overexpression prevents against HFD in mice, it is possible that this protection against obesity might be linked to scavenger receptor expression (33). Other reports have mentioned that Sirt6 has anti-inflammatory benefits via its effects on NF-κB mediated signalling. Although the BM-specific deletion of Sirt6 does not have a potent effect of whole body inflammation, it is likely that the local lesion site inflammation is affected.

Our findings with regard to Sirt6 affecting Msr1 and modified LDL uptake bolster the concept of future strategies of activating Sirt6 to alleviate cardiovascular diseases

and other age-related diseases.

FUNDING

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CONFLICT OF INTEREST

None

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5.3 Contributions to other published articles

The Sirt1 activator SRT3025 in *Apoe*$^{-/-}$ mice provides atheroprotection by reducing hepatic Pcsk9 secretion and enhancing LDL-R expression.

Miranda MX, van Tits LJ, Lohmann C, Arsiwala T, Winnik S, Tailleux A, Stein S, Gomes AP, Suri V, Ellis JL, Lutz TA, Hottiger MO, Sinclair DA, Auwerx J, Schoonjans K, Staels B, Lüscher TF, Matter CM.

Journal:

Eur Heart J. 2014, *fasttrack* publication

PMID:

24603306

Contributions:

1. Experiments for Fig 5 A and B

2. Revisions

6. PERSPECTIVES

6.1 Role of Sirt6 in atherosclerosis

The current thesis focuses on investigating the effects of Sirt6 deletion on atherosclerosis. We have shown that heterozygous deletion of Sirt6 does not affect atherosclerosis in *Apoe*$^{-/-}$ mice compared to control mice. However, atherosclerosis was increased in *Apoe*$^{-/-}$ mice transplanted with Sirt6-deficient bone marrow compared to *Apoe*$^{-/-}$ mice transplanted with wild-type bone marrow. These findings show that Sirt6 indeed plays a pro-atherosclerotic role in macrophages.

Although others and we have demonstrated that Sirt6 deletion increases atherosclerosis, the role of Sirt6 overexpression on atherosclerosis remains to be investigated [73, 83]. Constitutive Sirt6 overexpressing mice exhibit reduced LDL-c when placed on a high-fat diet [62] and Sirt6 also reduces LDL-c by means of regulating PCSK9 [91]. This suggests that Sirt6 overexpression may be athero-protective through an increase in hepatic LDL-R and a decrease in plasma PCSK9 and LDL-c levels. Sirt6 is also known to improve other risk factors of the metabolic syndrome such as insulin resistance, triglyceride synthesis and hepatic steatosis. Thus, the role of Sirt6 overexpression needs to be investigated in the context of both an *Apoe*$^{-/-}$ model and an *Ldlr*$^{-/-}$ model to distinguish between the atheroprotective effects of non-HDL lipoproteins and glucose metabolism. We anticipate that atherosclerotic effects of Sirt6 overexpression in *Apoe*$^{-/-}$ mice are different from *Ldlr*$^{-/-}$ mice.

Contrary to our data, others have shown an increase in atherosclerosis in heterozygous mice on an *Apoe*$^{-/-}$ background. Similarly, shRNA-mediated knockdown

of Sirt6 causes an increase in vascular endothelial dysfunction and atherosclerosis in *Apoe*$^{-/-}$ mice. However, since both studies show a change in Sirt6 activity, the discrepancy in our findings may lie in the difference in design of our *Sirt6*$^{-/-}$ mice experiments. Of note, similar to publications of Sirt1 deletion and the mechanism of atherosclerosis in *Apoe*$^{-/-}$ mice, studies of Sirt6 and atherosclerosis in *Apoe*$^{-/-}$ mice demonstrate different protagonist cells such as NK cells in Sirt6 heterozygous mouse and vascular cells in shRNA-mediated knockdown of Sirt6, while we show Msr1 in macrophages. These findings demonstrate novel roles of Sirt6 in NK cells, vascular endothelial cells and macrophages. Thus, Sirt6 might play a diverse role in different cell types and accordingly lack of Sirt6 functions to be a pro-atherosclerotic protagonist in *Apoe*$^{-/-}$ mice.

6.2 Role of Sirt6 in macrophage-mediated atherosclerosis

My study of bone marrow-specific Sirt6 deletion in atherosclerosis prone *Apoe*$^{-/-}$ mice found that deficiency of Sirt6 in BM cells is proatherosclerotic.

There are limited studies linking Sirt6 to improved lipid metabolism and even fewer studies investigating the role of Sirt6 and macrophages. Our findings that Sirt6 in macrophages is sufficient for atheroprotection by regulating the Msr1 expression highlights a novel aspect of Sirt6 being involved in macrophage lipid metabolism. Macrophages are important contributors to atherogenesis. Since we have demonstrated that Sirt6 regulates an important macrophage lipid influx moiety – Msr1, it would be interesting to further assess whether Sirt6 also plays a role in other aspects of macrophage lipid metabolism and plaque progression.

For example, efferocytosis describes the process by which macrophages

remove dying or dead cells by phagocytosis (Figure 8). Since macrophages clear cell debris, progression of fatty streaks to advanced plaques with necrotic core has been linked to a defect in macrophage efferocytosis. PPARγ acts as a transcription factor for many genes involved efferocytosis. Sirt6 overexpression downregulates PPARγ gene expression [62]. Similarly, miR-34a is known to negatively regulate efferocytosis [92]. Sirt6 is a miR-34a target downregulated during differentiation [93]. Hence, the effects of PPARγ or miR-34a on efferocytosis might be Sirt6-dependent and worthwhile to be investigated.

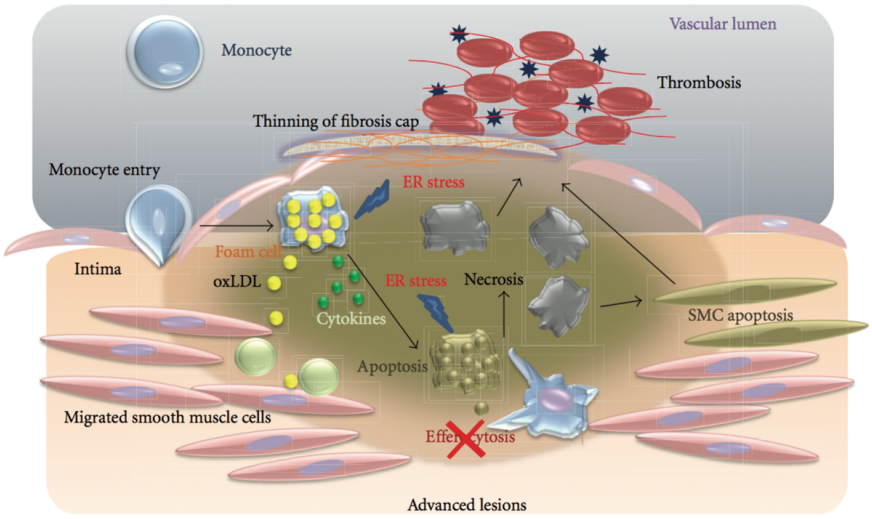

Figure 8. Cellular interactions of macrophages in advanced lesions: Foam cells accumulate in the intima and trigger inflammatory responses. This causes increased ER (endoplasmic reticulum) stress that in turn leads to macrophage apoptosis and defective efferocytosis. The apoptotic cells become necrotic cells, contributing to necrotic core formation. Necrosis of macrophages and SMCs decrease collagen synthesis, reducing the collagen content of the fibrous cap, triggering plaque rupture and thrombosis. (Adapted from Gui et al., Mediators of Inflammation, 2012) [94].

6.3 Targeting monocytes and macrophages towards plaque regression

The **leukocyte adhesion cascade** comprises steps that facilitate the entry of monocytes into the arterial intima, including the adhesion of monocytes to the endothelium (depends on the upregulation on activated endothelial cells of molecules), rolling which is mediated by selectins, activation (which is mediated by chemokines) and arrest (which is mediated by integrins) [95]. Sirt6 reduced the expression of inflammatory cytokines such as P-selection, VCAM1 and ICAM1. It is possible that Sirt6 provides athero-protection by downregulating these cytokines at the different stages plaque formation.

For patients with coronary or cerebral atherosclerosis, understanding atherogenesis is important in terms of preventing or decreasing plaque formation. However, plaque regression would be the ultimate goal. My studies have proven the Sirt6 is important for experimental atherogenesis. It will also be interesting to study the role of Sirt6 in aspects of plaque regression. Atherosclerosis regression is typically associated with a decrease in atherosclerotic plaque size with a marked reduction in lipid levels, immune cells and inflammatory gene expression. Transcriptomic profiling of macrophages isolated from an aortic transplantation mouse model of progressing and regressing plaques identified >700 genes that are differentially regulated [96]. These genes included the retention factors such as netrin 1, semaphorin 3E and cadherins, which reduce efferocytosis and emigration of the macrophages from the plaques [97].

To come up with effective therapies targeting plaque macrophages, we must

consider both aspects–plaque progression and plaque regression. The timing of the drug or treatment is equally important. The best approach would be to abrogate early plaque development, when efferocytosis is most efficient, and the apoptosis of accumulating plaque cells. To retard plaque progression, agents such as Il-10 or LXR agonists, have been tested [98, 99]. Along with having benefits in plaque progression, these agents have additional effects such as reducing inflammation (in the case of Il-10 and LXR agonists) or promoting cholesterol efflux (in the case of LXR agonists). It is interesting to note that both LXR and IL-10 are Sirt1 targets. It would be interesting to check whether Sirt6 also regulates these and if the plaque regressive effects are actually prompted by a shift in Sirtuins activity locally. Another possible way to determine if Sirt6 is indeed involved in plaque regression is by doing a ChIP-sequencing of macrophages isolated from an aortic transplantation mouse model of progressing and regressing plaques and assessing for common targets by transcriptomic profiling.

While the concept of plaque regression in patients appears appealing, translation of experimental findings to patients with atherosclerosis appears challenging given species differences with different metabolism, time course of atherogenesis and potential side- and off-target effects of such interventions.

6.4 Role of Msr1 in other diseases – A novel link to the role of Sirt6?

We have shown that Sirt6 modulates Msr1 expression and that less Sirt6 causes an increase in risk to atherosclerosis. However, Msr1 also plays a role in different

diseases. For example, Msr1 may enhance the pathogenesis of neurodegeneration [100] and the production of neurotoxins upon central nervous system (CNS) injury [101]. In line with this, *Sirt6*$^{-/-}$ mice show an increase in DNA damage, suggesting a physiological role of Sirt6 in maintaining integrity of neural cells [102].

Msr1 has also been shown to play a role in retinitis pigmentosa (retinal degeneration). Retinal pigment epithelium expresses Msr1 receptor, where an increase in causes an increase in retinal degeneration [103]. Conversely, retinal Sirt6 expression has been shown to be required for normal functioning of the retina [104].

Msr1 is also associated with prostrate cancer. Germline mutation of Msr1 increases risk to prostate cancer [105], while polymorphisms in Msr1 are also linked to increased incidence of prostate cancer as seen from epidemiological studies [106, 107]. While overexpression of Sirt6 increases cell viability in prostate cancer [108]. However, studies have not yet addressed the role of Sirt6 and Msr1 in CNS injury, retinitis pigmentosa or prostate cancer. Thus, further studies are required to investigate these links between Sirt6 and Msr1 in these diseases.

6.5 Sirtuin activating compounds

To take advantages of the beneficial effects sirtuins in experimental contexts, there is a growing interest in the development of novel small molecules modifying sirtuin activity. Since Sirt1 was the first sirtuin to be investigated, most of the available sirtuin activating compounds (STACs) targeted Sirt1. Resveratrol – a naturally occurring compound was the first STAC that was identified by screening for

deacetylation of synthetic peptidyl substrates labeled with a chemical fluorophore group [109, 110] which was later shown to be non-specific screening method [109].

The discovery of natural STACs such as resveratrol was a proof-of-concept that prompted screening for synthetic Sirt1 activators that were more potent, soluble and bioavailable [109]. STACs such as SRT1720 and SRT2104 have been tested in human clinical trials for different indications such as psoriasis, skin cancer, lymphoma among others [110, 111] – including cardiovascular diseases (see below).

The experimental beneficial effects of Sirt1 activation prompted the search for Sirt6 activators. Unfortunately, there are no commercially STACs specific for Sirt6 available however recently a patent application have been filed for a Sirt6 activator where the compound increases the deacetylation activity of Sirt6. The compound binds to Sirt6 and reduces the Km of Sirt6 for a substrate, thereby increasing the deacetylase activity of Sirt6 (US patent application number: US 13/516,198). All the experiments in the patent application file were *in vitro* chemical assays and it would be prudent to assess its activity in cell culture assays.

In addition to exogenous chemical activators of Sirt6, endogenous activators of Sirt6 have been identified. The nuclear lamins, in conjunction with their interacting partners, form a complex network of proteins that are critical for the mechanical support of the nuclear envelope, and maintenance of proper chromatin organization and transcriptional regulation [112]. It was recently shown that lamin A directly binds and activates Sirt6 toward histone deacetylation. It thus functions as

an endogenous activator of Sirt6 and promotes Sirt6-mediated downstream effects upon DNA damage [113].

This study reveals that Sirt6 activity can be stimulated by both novel exogenous and endogenous means.

6.6 STACs in cardiovascular disease

The available STACs have been tested in numerous animal models and have been tested in clinical trials for their beneficial effects. Studies have shown that in diet-induced obese and genetically obese mice, selective STACs such as SRT1460, SRT2183 and SRT1720 lowered plasma glucose and increased mitochondrial capacity [114]. SRT3025 treatment reduced atherosclerosis by lowering plasma levels of LDL-c and total cholesterol, increasing LDLR and decreased secretion of Pcsk9 [115]. Since Sirt6 overexpression reduces LDL-c levels, it is possible that Sirt6 activation would also protect against atherosclerosis. Alternatively, since Sirt6 knockout mice are hypoglycemic and overexpression improves insulin sensitivity, Sirt6 activation may also be beneficial in treating metabolic syndrome – a risk factor of atherosclerosis. Given these putative protective effects, there may be a high interest for pharmaceutical companies to develop STACs targeting Sirt6.

6.7 Alternative pathways of sirtuin activation: increasing NAD^+ levels

The activity of sirtuins is controlled by the cellular NAD^+: NADH ratio, mainly on the

availability of its co-factor, NAD^+ that is essential for sirtuin deacetylase activity. This NAD^+: NADH ratio is tightly controlled by changes in cellular metabolism [116]. This may explain why caloric restriction increases sirtuin activity; caloric restriction lowers metabolism and oxidative stress, increasing availability of NAD^+ [117]. Hence, a natural way to increase the activity of sirtuins is by caloric restriction.

The modulation of intracellular NAD^+ levels is another method to increase sirtuin activity and mimic its beneficial effects. This can be achieved by two different strategies – increasing NAD^+ biosynthesis or decreasing NAD^+ consumption. During the biosynthesis of NAD^+ within cells, the enzyme nicotinamide phosphoribosyltransferase (NAMPT) catalyses the rate limiting step of converting nicotinamide to nicotinamide mononucleotide (NMN) and pyrophosphate [118]. NMN is then converted to NAD^+ by the isoenzyme nicotinamide mononucleotide adenylyltransferase (NMNAT).

Currently, few studies have addressed the modulation of NAD^+ levels by supplementation of tryptophan and nicotinic acid (NA). Tryptophan is the main precursor required for the *de novo* biosynthesis of NAD+, while nicotinic acid is another NAD+ precursor required by the Preiss-Handler pathway [119]. These are a few studies where supplementation with these precursors influence NAD^+ levels and in particular affect sirtuin activity [120]. Both NA and NAM can lead to higher NAD^+ levels, however this effect seems to be tissue-specific.

In addition to boosting NAD^+ levels through administration of NAD^+ precursors, NAD^+ levels can also be artificially modulated by changing the activity of rate-limiting enzymes in NAD^+ biosynthesis. The modulation of NAMPT activity has

an impact on NAD^+ levels in all mammalian cells and in majority of the cases this is accompanied by an increase in sirtuin activity which can be linked to an increase in NAD^+ levels promoted by differential NAMPT activity [119, 121].

7. CONCLUSIONS

My PhD project demonstrates that specific loss of Sirt6 in BM-derived cells in proatherosclerotic by upregulating Msr1 expression, without affecting systemic inflammation. It emphasizes the importance of BM-derived cells in atherogenesis. It also spotlights the contribution of the local aortic mmLDL uptake by scavenger receptors in atherogenesis. Most importantly, it supports the concept of Sirt6 providing experimental atheroprotection and underlines the attractive perspectives to test this notion in patients with atherosclerotic disease.

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10. CURRICULUM VITAE

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EDUCATION

University of Zurich, Center for Molecular Cardiology 2013-November 2016
Doctor of Philosophy in Integrative Molecular Medicine
- Dissertation title `The role of Sirtuin 6 in atherosclerosis'

University of Oxford, Dept. of Pharmacology 2010-2011
Masters of Science in Pharmacology
- Dissertation title ` Investigation of the effects of IMPase inhibitor- Ebselen on the central 5-HT function'

University of Oxford, Said Business School 2010-2011
Building a Business certificate program
- Program focus on marketing process, intellectual property, financial projections

University of Mumbai, MET Institute of Pharmacy 2006-2010
Bachelor of Pharmacy

WORK AND RESEARCH EXPERIENCE

Research Associate at the University of Zurich, Center for Molecular Cardiology 2013-2016
-Study disease progression with *in vivo* animal models
-Flow cytometry to assess macrophage and bone marrow subpopulations
-Immunofluorescence and other staining methods, cell-based assays

Junior Research Fellow at the Tata Institute of Fundamental Research 2012-2013
-Working on project `Investigating the brain circuits activated in response to postnatal and adult life stress'

Teaching assistant at the University of Oxford 2010-2011
-*In vivo* and *in vitro* based practicals demonstrating the pharmacology of drugs on cardiovascular and respiratory system

M.Sc. Dissertation at the University of Oxford 2011
-Assess the neurobiochemical effects of Ebselen on the brain using HPLC
-Study the activation of central pathways (Behavioural studies, gene expression analyses)

Trainee at Abbott India Limited for 3 months 2009
-Quality control analysis of finished drugs
-Microbial testing and handling bacterial cultures
-Daily quality control and conformance reports

EXPERIMENTAL TECHNIQUES

- Flow cytometry, ELISA, ex vivo assays
- *In vivo* animal work
- Immunoprecipitation
- Staining: Immunohistochemistry, immunofluorescence, and confocal microscopy
- *In vitro* cell culture skills (siRNA transfections, adenovirus transduction)
- Molecular biology techniques like western blotting, quantitative PCR

AWARDS AND PRIZES

Young Investigator Award in Vascular Biology at the European Society of Cardiology Conference 2016
Best poster prize at the 12th ZHIP symposium, University of Zurich 2015
Best poster prize at Indian Pharmaceutical Association Congress 2010

PRESENTATIONS

Oral presentation at the European Atherosclerosis Society, Innsbruck 2016
Title `Bone marrow-specific Sirt6 deletion increases atherogenesis by increasing macrophage scavenger receptor 1'

Oral presentation at the European Society of Cardiology, Rome 2016
Title `Bone marrow-specific *Sirt6* deletion enhances atherosclerosis and foam cell formation via an increase in macrophage scavenger receptor 1 expression'

Oral presentation at the 12th ZHIP symposium, University of Zurich 2016
Title `Bone marrow-specific *Sirt6* deletion enhances atherosclerosis by increasing macrophage scavenger receptor 1 expression'

Oral presentation at Wagi Science Panorama seminar, University of Zurich 2016
Title `Bone marrow-specific *Sirt6* deletion enhances atherosclerosis and foam cell formation via an increase in macrophage scavenger receptor 1 expression'

Poster presentation at the 11th ZHIP symposium, University of Zurich 2015
Title: `Sirt6 decreases foam cell formation via macrophage scavenger receptor 1 – implications for atherogenesis'

Poster presentation at the Indian Pharmaceutical Association, Mumbai 2010
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PUBLICATIONS

- T. Arsiwala, L. Tits, MX. Miranda, K. Nussbaum, J. Weber, P. Blyszczuk, S. Stivala, JH. Beer, M. Greter, B. Becher, MO. Hottiger, R. Mostoslavsky, U. Eriksson, B. Staels, CM. Matter, TF. Lüscher. Bone marrow-specific *Sirt6* deletion increases foam cell formation and enhances atherosclerosis by increased macrophage scavenger receptor 1 expression (In preparation)
- MX. Miranda, LJ. Tits, C. Lohmann, T. Arsiwala, S. Winnik, A. Tailleux, S. Stein, AP. Gomes, V. Suri, JL. Ellis, TA. Lutz, MO. Hottiger, DA. Sinclair, J. Auwerx, K. Schoonjans, B. Staels, TF. Lüscher, and CM. Matter (2014). The Sirt1 activator SRT3025 provides atheroprotection in Apoe $^{-/-}$ mice by reducing hepatic Pcsk9 secretion and enhancing Ldlr expression. European Heart Journal DOI: 10.1093/eurheartj/ehu095
- Antoniadou, T. Fowler, E. Cardici, T. Arsiwala, T. Sharp (2013). Effect of the putative lithium-mimetic ebselen on 5-HT neurochemistry in the mouse brain. Conference Paper in European Neuropsychopharmacology DOI: 10.1016/S0924-977X (13) 70571-6

ACTIVITIES

Member of Vision 2020 a personal perspective, University of Zurich
Member of Indian Pharmaceutical Association

OTHER SKILLS AND INTERESTS

IT Skills: Microsoft office, Adobe Illustrator and Photoshop, Prism GraphPad data analyses
Languages: English-proficient, French and German-Basic
Hobbies: Nature macro photography, word games like scrabble, kayaking